Meiotic targets of the Ras/cAMP/PKA pathway during the regulation of spore formation in *Saccharomyces cerevisiae*

Dissertation

zur

Erlangung des Doktorgrades

der Naturwissenschaften

(Dr. rer. Nat.)

dem

Fachbereich Biologie

der Philipps-Universität Marburg

vorgelegt von

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Marburg an der Lahn, Oktober 2017

Dissertation accepted from the Department of Biology of the Philipps-Universität Marburg on_____

First reviewer: PD Dr. Christof Taxis, second reviewer: Prof. Dr. Hans-Ulrich Mösch

Date of the dissertation_____

The investigations of the present work were carried out under the the supervision of Dr. Christof Taxis during the period February 2012 to March 2016 at the Philipps-Universität Marburg.

Declaration

I hereby declare that I have prepared my dissertation with the title "Meiotic targets of the Ras/cAMP/PKA pathway during the regulation of spore formation in *Saccharomyces cerevisiae*" independently, without unauthorized help and that I have used none other than the sources and assistance expressly indicated by me.

Currently the dissertation has not yet been submitted to any other institution or a similar form and has not yet served any other purpose.

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(Place, Date) (Massimiliano Muscó)

TO MY TENACITY AND PERSEVERANCE

NOTHING IS IMPOSSIBLE..

Summary

Sporulation in *Saccharomyces cerevisiae* occurs in response to starvation for nutrients and in presence of a non-fermentable carbon source like potassium acetate which lead diploid yeast cells to undergo meiosis and subsequently to package the haploid nuclei in ascospores. The number of formed spores is between one and four and depends on the availability of nutrients, the more nutrients are available the higher is the number of spores per ascus. This regulation of the spore numbers is called spore number control (SNC) and occurs at the yeast centrosome called spindle pole body (SPB). A meiotic placque (MP) which works as scaffold to initiate the synthesis of the prosporemembrane, is composed by three essential proteins Mpc54, Mpc70 and Spo74. The regulation of spore number is controlled by nutrients which according to the concentration of potassium acetate (KOAc) regulate the amount of MP proteins and by the spindle polarity which is the basis for adaptation of gamete numbers during meiosis, the age of the SPBs indeed is crucial for the selection of the SPBs to modify and that will become spores. The protein Ady1 is phosphorylated by the kinase PKA *in vitro*. Ady1 has a genetic interaction with the SPB components and localizes all the MP components to the SPBs, it is responsible then of the formation and the activity of the MP. The nutrient-responsive Ras/cyclic AMP (cAMP)/protein kinase A (PKA) pathway influences proliferation, carbohydrate metabolism, entry into meiosis and gamete numbers in response to nutrients in *Saccharomyces cerevisiae*. In this study, it was shown how Ras/cAMP/PKA signal transduction pathway influences the adjustment of spore numbers to external nutrients. Specifically, low PKA activity increased the spore formation by increasing the abundance of two MP proteins Mpc70 and Spo74 plus Ady1 when the source of potassium acetate is poor. The approach which has been used to induce high PKA prevented spore formation, many cells did not reach the meiotic divisions. Nevertheless, by this method it was possible to see that the Mpc54 protein encoded by the early gene *MPC54* is affected in its abundance as well. My assays revealed that all these players are *in vivo* targets of PKA. Mpc54, Mpc70 as well as the meiotic protein Ady1 could be indirectly targets of PKA. The data suggested that Spo74, is a direct target of PKA and this direct regulation contributed to spore number control. PKA activity as well affected the timing of meiotic entry, cells entered meiosis earlier when PKA was reduced. The collected data indicated that inactivation of the transcription factor Nrg1 reduces spore formation. Yet, high PKA activity affected negatively the plasma membrane localization of Ras proteins during vegetative growth and sporulation. Finally, it has been shown that the activity of PKA controls the activity of other components like the nucleolar

protein Fob1, the regulatory subunit of Snf1 called Snf4 and the nicotinamidase Pnc1. Overall, the findings imply the involvement of the Ras/cAMP/PKA pathway in the regulation of gamete numbers during yeast meiosis.

Zusammenfassung

Sporulation in *Saccharomyces cerevisiae* entsteht durch Nährstoffmangel in Gegenwart einer nicht-fermentierbaren Kohlenstoffquelle wie Kaliuacetat, welches diploide Hefezellen veranlasst die Meiose einzuleiten und darauf folgend haploide Nuklei in Ascosporen zu verpacken. Die Anzahl der gebildeten Sporen liegt zwischen einer und vier und hägt von der Verfügbarkeit von Nährstoffen ab. Je mehr Nährstoffe verfügbar sind, desto höher ist die Anzahl von Sporen pro Ascus. Diese Regulierung der Sporenzahl wird als '*spore number control*' (SNC) bezeichnet und tritt am Hefe-Zentrosom, genannt '*spindle pole body*' (SPB), auf. Die meiotische Platte (MP), welches als Gerüst dient um die Synthese der Prosporenmembran zu initiiren, setzt sich aus den drei essentiellen Proteinen Mpc54, Mpc70 und Spo74 zusammen. Die Regulierung der Sporenzahl wird sowohl durch Nährstoffe kontrolliert, welche in Abhängigkeit der Konzentration von Kaliumacetat (KOAc) die Menge der MP-Proteine regulieren, als auch durch die Spindelpolarität, welche die Grundlage für die Adaptation der Gametenzahl während der Meiose ist. Das Alter der SPBs ist entscheidend für die Auswahl, welche der SPBs zu Sporen werden. Das Protein Ady1 wird *in vitro* durch die Kinase PKA phosphoryliert. Ady1 hat eine genetische Interaktion mit SPB-Komponenten und lokalisiert alle MP-Komponenten an den SPBs, wodurch es für die Formierung und Aktivität des MP verantwortlich ist. Der nährstoffabhängige Ras/zyklisches AMP (cAMP)/Proteinkinase A (PKA) Signaltransduktionskaskade beeinflusst die Proliferation, den Kohlenhydratstoffwechsel, den Eintritt in die Meiose und die Gametenzahl in Abhängigkeit von Nährstoffen in *Saccharomyces cerevisiae*. In dieser Arbeit wurde gezeigt, wie die Ras/cAMP/PKA Signaltransduktionskaskade die Regulierung der Sporenzahl durch externe Nährstoffe beeinflusst. Besonders eine hohe PKA-Aktivität reduziert die Sporenbildung durch eine Reduzierung der Abundanz der Komponente Ady1. Eine niedrige PKA-Aktivität erhöht die Sporenbildung durch die Erhöhung der Abundanz von zwei MP-Proteinen und Ady1 bei geringer Natriumacetatkonzentration. Die Methode, welche angewandt wurde um die PKA zu induzieren, verhinderte die Sporenbildung. Viele Zellen durchzogen keine meiotische Teilungen. Jedoch konnte mit dieser Methode gezeigt werden, dass Mpc54, codiert durch das frühe Gen *MPC54*, auch in seiner Abundanz beeinflusst wird. Meine Untersuchungen zeigten, dass alle diese Komponenten *in vivo* ein Ziel der PKA sind. Mpc54, Mpc70 als auch das meiotische Protein Ady1 könnten indirekte Ziele der PKA sein, wobei die Daten darauf hindeuten, dass Spo74 ein direktes Ziel der PKA ist und diese direkte Regulierung zur Kontrolle der Sporenzahl beiträgt. Die PKA-Affinität beeinflusste auch die zeitliche

Regulierung des Eintritts in die Meiose, denn die Zellen traten früher in die Meiose ein wenn die PKA-Aktivität rediziert war. Die gesammelten Daten deuten daraf hin, dass eine Inaktivierung des Transkriptionsfaktors Nrg1 die Sporenbildung reduziert. Jedoch beeinflusste eine hohe PKA-Aktivität negativ die Plasmamembranlokalosierung von Ras-Proteinen während des vegetativen Wachstums und der Sporulation. Es konnte gezeigt werden, dass die Aktivität der PKA auch die Aktivität weiterer Komponenten wie dem Kernprotein Fob1, der regulatorischen Untereinheit von Snf1, genannt Snf4 und der Nikotinamidase Pnc1 beeinflusst. Zusammenfassend erklären die Beobachtungen die Beteiligung des Ras/cAMP/PKA Pfades bei der Regulierung der Gamentenzahl während der Meiose der Hefe.

Abbreviations and technical terms

(d) dH2O	(fully) deionized water
(K) bp	(kilo) base pairs
AD	activation domain
AMP	adenosine monophosphate
Amp	Ampicillin
APS	ammonium persulfate
ARS	autonomous replication sequence
ATP	adenosine triphosphate
bPAC	photoactivated adenylyl cyclase from bacteria
cAMP	cyclic adenosine monophosphate
carnitine shuttle	acyl carnitine / carnitine transport system
commitment	Entry into meiosis; Time between the end of Prophase I and the start of Meiosis I
CO_2	Carbon dioxide
C-terminal	Carboxyterminal
DAPI	4 ', 6-diamidine-2-phenylindole
DBD	DNA-bindind domain
DIC	Differential interference contrast
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Ditiothreitol
ECL	enhanced chemoluminescence
EDTA	Ethylenediamine tetraacetic acid
et al.	“And other”
EtOH	Ethanol
GAD	Gal4 activation domain
Gal	Galactose
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
Glc	Glucose
GTP	Guanosine triphosphate
HA	Haemagglutinin epitope

HCl	Hydrochloride
HCO_3^-	Bicarbonate
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
KOAc	Potassium acetate
LB	Lysogeny Broth complex nutrient medium for *E. coli*
LexA	bacterial protein used as DBD in combination with Gal4 AD
LFM	low fluorescent medium
m	milli
mM	millimolar
M	Concentration of substance in mol / L
MAPK	Mitogen activated protein kinase
Mating type (Mat)	Crossing / or sex type
Meiosis I	First meiotic division
Meiosis II	Second meiotic division
min.	minute
MP	Meiotic plaque
Myc	Myc epitope
NAD	Nicotinic acid amide dinucleotide
NADH	Hydrated form of nicotinic acid amide dinucleotide
$NaHCO_3$	Sodium hydrogencarbonate
Na_2CO_3	Sodium carbonate
N terminal	Amino terminal
NTS	nontranscribed regions
OD	optical density
ONPG	ortho-nitrophenyl-β-galactosidase
ORF	open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Poylmerase chain reaction
PEG	Polyethylene glycol
pH	power of hydrogen
PKA	Protein kinase A
Primer	Oligonucleotide

pTEV+	Gen, which encodes the modified TEV protease. P: p14 D122Y, +: STOP-Codon in place 234
Raff	Raffinose
RFB	replicative fork barrier
RNAse	Ribonuclease
RP	ribosomal protein
RT	Room temperature
SC	"Synthetic complete" synthetic nutrient medium for S. cerevisiae
SDS	sodium dodecyl sulfate
SORB	sorbitol
SPB	spindle pole body
SPO	Sporulation
Taq	Thermus aquaticus
TBS (T)	Tris buffered saline solution (with Tween 20)
TC	Cell + growth
TCA cycle	Citrate cycle
TDegF	TEV protease activatable degron with phenylalanine as N terminal amino acid after proteolysis by TEV protease and SF3B (383-424) interacting with high affinity with p14
TEMED N, N, N ', N',	Tetramethylenediamine
TEV	tobacco etching virus
TIPI	TEV-protease induced protein instability
Tris	tris-(hydroxymethyl)-aminomethane
Tween	polyoxyethylene sorbitan monolaurate
UV	Ultraviolett
O/N	Over Night
v/v	Volume per Volume
w/v	Mass per Volume
wt	Wild type
YP	"Yeast extract + Peptone"
YPD	"Yeast extract + Peptone + Dextrose"
YPG	"Yeast extract + Peptone + Glycerol"
Z-Stacking	Recording of several images in Z direction
°C	Degree centigrade

%	Percent
α	alpha
µ	micro
Δ	Delta (Characterizes the deletion of a gene)

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1 Introduction

1.1 The baker's yeast *Saccharomyces cerevisiae*

The species of yeast *Saccharomyces cerevisiae* belongs to the phylum of the Ascomycota. It is famous for being utilized for baking, brewing and winemaking, furthermore is one of the most used eukaryotic organisms in molecular and cell biology. It is easy to grow and breed in culture media, its doubling time is short, about 90 min and most important its genome has been completely sequenced (Goffeau et al., 1996). Many proteins found in yeast share similar sequences with proteins from other eukaryotes. By investigating the function of a given protein in yeast is possible to gain insight into the protein's function in higher eukaryotes. Studying the biology of this yeast has enabled scientists to work out the connections between genes and proteins, and the functions they carry out in our cells. Recently the new frontiers of the research aim to study with the help of such model organism something more important than the function of a single gene or protein, this is the "system level". It means to study the function of the whole genes and proteins as they act to maintain metabolism and cellular homeostasis under a great diversity of environmental conditions and to provide for the regulation and organization of reproduction, cellular growth, and development (Botstein and Fink, 2011). Haploid and diploid stages in absence of carbon source arrest their cell cycle in G1 phase. Yeast cells can reproduce asexually in presence of nutrients. They form through mitotic cell divisions a small daughter cell called bud, which is cut off from the mother. Haploid cells can reproduce sexually. The two sex Mat alpha and Mat a recognize each other by pheromone signals and receptors (Leberer et al., 1997) and merge together to form diploids through plasmogamy and karyogamy.

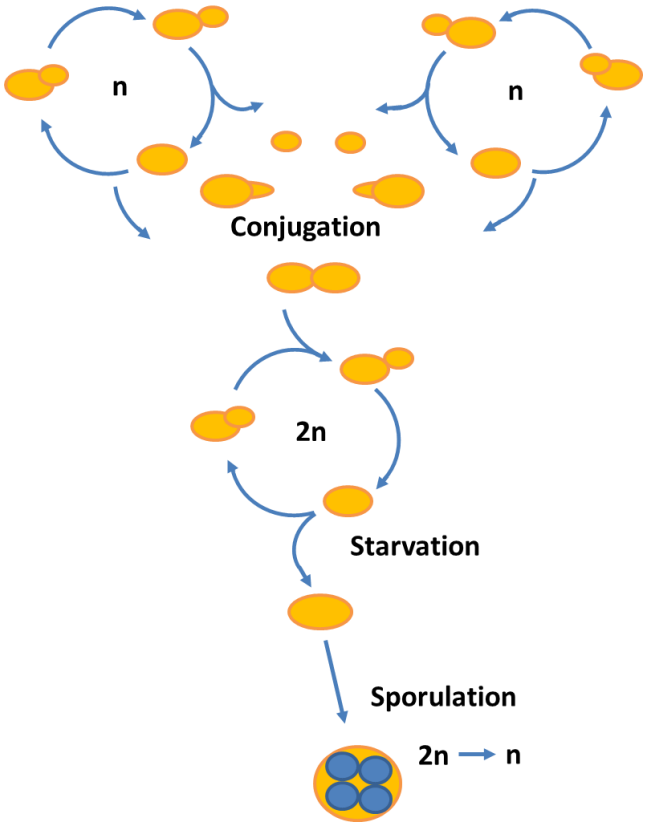

Figure 1: Life cycle of *Saccharomyces cerevisiae*

Yeast can reproduce in different ways, either haploid or diploid cells can multiply by budding, haploids produce diploids by conjugation while a diploid cell according to the nutritional status give rise to the production of an ascus with up to four haploid spores. This process is called sporulation

The reverse transition from diploid to haploid state is called sporulation (Figure 1). In absence of nutrients like glucose, nitrogen, sulphate, phosphate and in presence of a non-fermentable carbon source sporulation, which is coupled to meiotic cell divisions leads to the formation of four haploid cells called spores. Two of them have mating type alpha while the other two have mating type a.

1.2 The sporulation in *S. cerevisiae*

1.2.1 The formation of spores

Sporulation process in *Saccharomyces cerevisiae* is coupled to meiosis and leads to the formation of an ascus, which can contain up to four haploid spores (Esposito and Klapholz, 1981). The initiation of sporulation occurs when nutrients like glucose, nitrogen, sulphate or phosphate are missing. A small quantity of glucose could repress sporulation, indeed even a concentration between 0.2 and 0.5% inhibits the process (Honigberg and Purnapatre, 2003). A nutrient, which permits cells to sporulate is the non-fermentable carbon source potassium

acetate (KOAc). Sporulation guarantees genetic variability due to the combination to meiosis, which provides recombination between the homologous chromosomes. In the initial meiotic S-phase, DNA replicates (Figure 2). In the successive step, in prophase one, pairing of homologous chromosomes and recombination takes place. Afterwards two meiotic divisions occur and haploid chromosomes are formed. Yeast cells react to nutrients at all stages during sporulation but there is an important step in which there is a difference in the cellular response. It is called point of commitment and occurs between the end of prophase one and the first meiotic division (Friedlander et al., 2006; Simchen, 2009). Cells which have started the meiotic program and have not reached the point of commitment, in presence of specific nutrients can return to grow. When the point of commitment has been attained, cells complete meiosis even in presence of promoting-growth nutrients (Simchen et al., 1972).

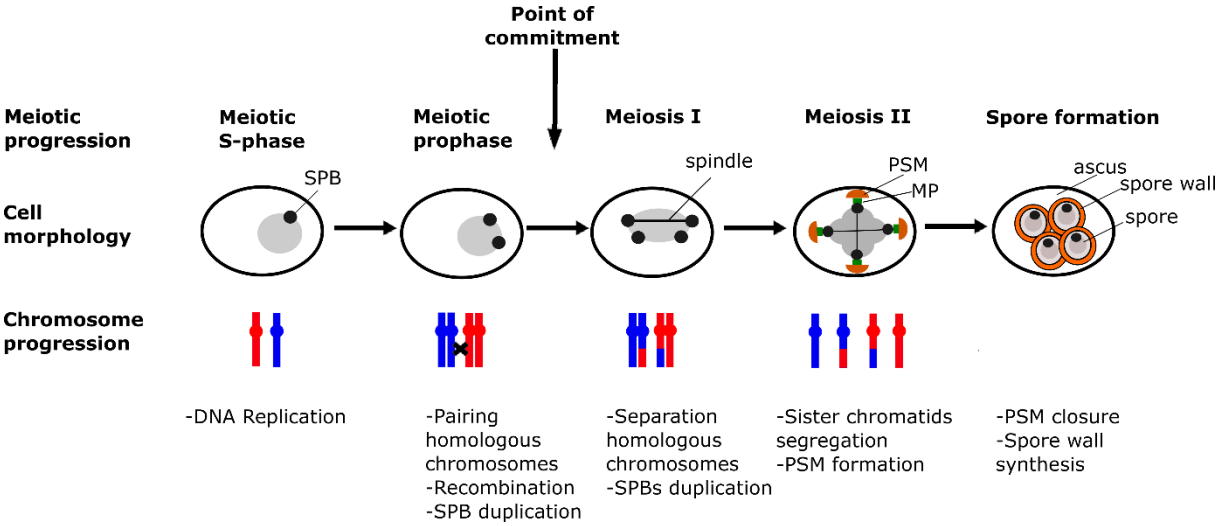

Figure 2: Sporulation *in Saccharomyces cerevisiae*

In the S-phase there is the replication of the DNA. During prophase pairing of homologous chromosomes and recombination occur. The spindle pole body (SPB) duplicates. In meiosis I there is the separation of homologous chromosomes and the two SPBs duplicate again. In meiosis II sister chromatids segregate and the formation of the prospore membranes (PSM) takes place by modification of the SPBs with the meiotic plaques (MP). After meiotic divisions the PSMs close including the haploid nuclei and part of the cytoplasm. Spore wall synthesis then begin between the two prospore membranes.

As in mitosis, meiosis in *S. cerevisiae* is closed (Byers and Goetsch, 1975) which means that nuclear membranes during meiosis remain so, they do not get resolved, therefore the meiotic divisions occur within the nuclear membrane (Heywood and Magee, 1976). To form spores first of all prospore membranes have to be generated around the nuclei. The resulted prospores then have to be enclosed by the so called spore wall. For the formation of the prospore membrane the cytoplasmic surface of the spindle pole body SPB is modified in order

to recruit and fuse vesicles responsible for the creation of the membranes (Moreno Borchart and Knop, 2003; Shimoda, 2004). The SPB is the sole microtubule organizing center in *S. cerevisiae*. It is equivalent to the mammalian centrosome and it is responsible of the spindles, therefore it has a role in the distribution of chromosomes during the cellular divisions. The SPB is composed by a central multilaminar structure embedded in the nuclear envelope therefore it has an inner face where meiotic spindles are nucleated and an external face or outer plaque where cytoplasmic microtubules are formed. In G1 cells one SPB is present inherited from last mitosis. It duplicates before meiosis I and as a result one spindle with the two respective poles is constituted, homologous chromosomes then divide. Before meiosis II the two SPBs duplicate and the resulting four SPBs are connected in pairs through the meiotic spindles. In the second meiotic division, the sister chromatids are then separated from each other. The initiation of spore formation initiates in the beginning of meiosis II when the modification of the SPB with the meiotic plaque (MP) occurs. The MP in meiosis II is a specific structure composed by the core protein Mpc54, Mpc70, Spo74 plus Ady4, which has a scaffold function for making the structure more stable. The outer plaque of the SPB in vegetative growth and meiosis I is composed by different proteins like Cnm67, Nud1 and the responsible for the organization of the cytoplasmic microtubules called Spc72 while in meiosis II the MP proteins replace Spc72 (Figure 3). The responsible for the recruitment of the MP components are Cnm67 and Nud1. This is an important step for the activation of the SPBs because the MP components can bind solely to Nud1 (Bajgier et al., 2001; Gordon et al., 2006, Knop and Strasser, 2000; Nickas et al., 2003).

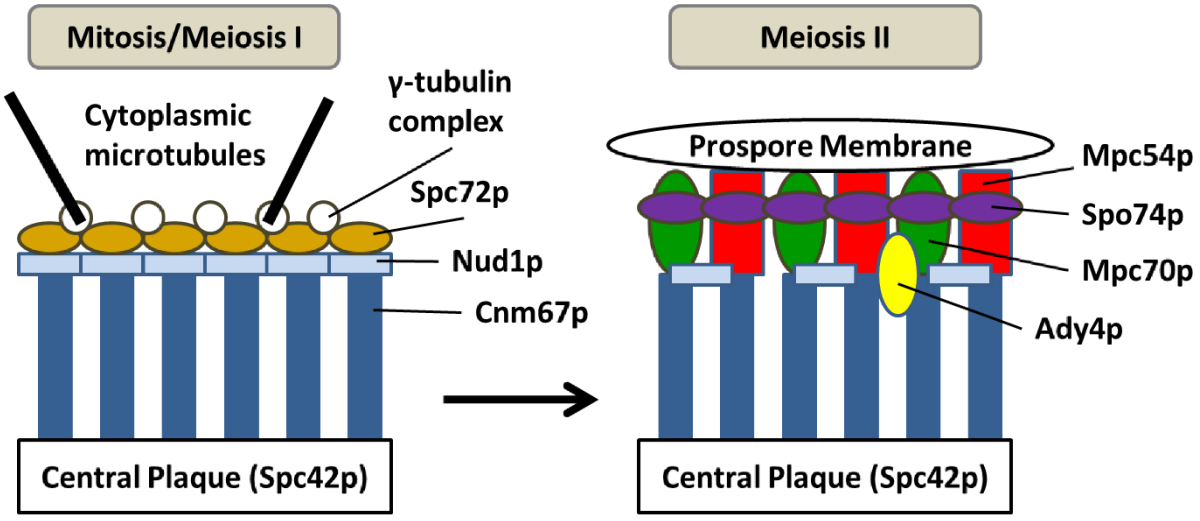

Figure 3: Organization of the meiotic plaque proteins within meiosis II

In mitosis and in meiosis I the gamma tubulin complex proteins and Spc72p ensure anchor of the cytoplasmic microtubules to the SPB. The new MP components are recruited by the SPB proteins

Nud1 and Cnm67. In the transition from meiosis I to meiosis II the composition of the SPBs components Spc42p, Cnm67 and Nud1 do not change (Modified from Neiman, 2005).

The prospore membrane is the future plasmamembrane of the developing spore (Moens, 1971; Moens and Rapport, 1971; Peterson et al., 1972) and is formed only on spindle-pole bodies which have previously been modified with a meiotic plate. The initial prospore membranes expand during their formation and engulf the forming haploid nuclei plus part of the cytoplasm of the mother when they finish meiosis II. Four haploid prospores are then formed. The initiation of the formation of the prospore membranes and the activation of all the SPBs is not simultaneous. The number of SPBs activated is limited meaning that it depends on the amount of MP proteins available (Taxis et al., 2005). The regulatory networks responsible for the MP formation are quite unknown. Two membranes surround the prospore membranes (Davidow et al., 1980; Peterson et al., 1972). After closure of the prospore membranes spore wall synthesis begins in the lumen of the two prospore membranes. The spore wall consists of four different layers. Components like mannan and beta-1,3-glucans are present in the first two layers, chitosan forms the third layer while the fourth layer is composed by dityrosine (Briza et al., 1988; Briza et al., 1986). Spores are resistant to several adverse conditions. The process of the spore formation occurs then in consequence of the meiotic divisions in order to protect the forming haploid nuclei against the external conditions (Coluccio et al., 2008; Smits et al., 2001).

1.2.2 Regulation of entry into sporulation by signal transduction pathways and the transition to meiotic division

The initiation of the sporulation depends on the nutrients present in the environment. A carbon source like glucose, essential nutrients like nitrogen, phosphate and sulphate have to be absent while the presence of a non-fermentable carbon source is requested. Characteristic of the yeast cells is to sense such nutritional conditions and to react in the proper way (Figure 4).

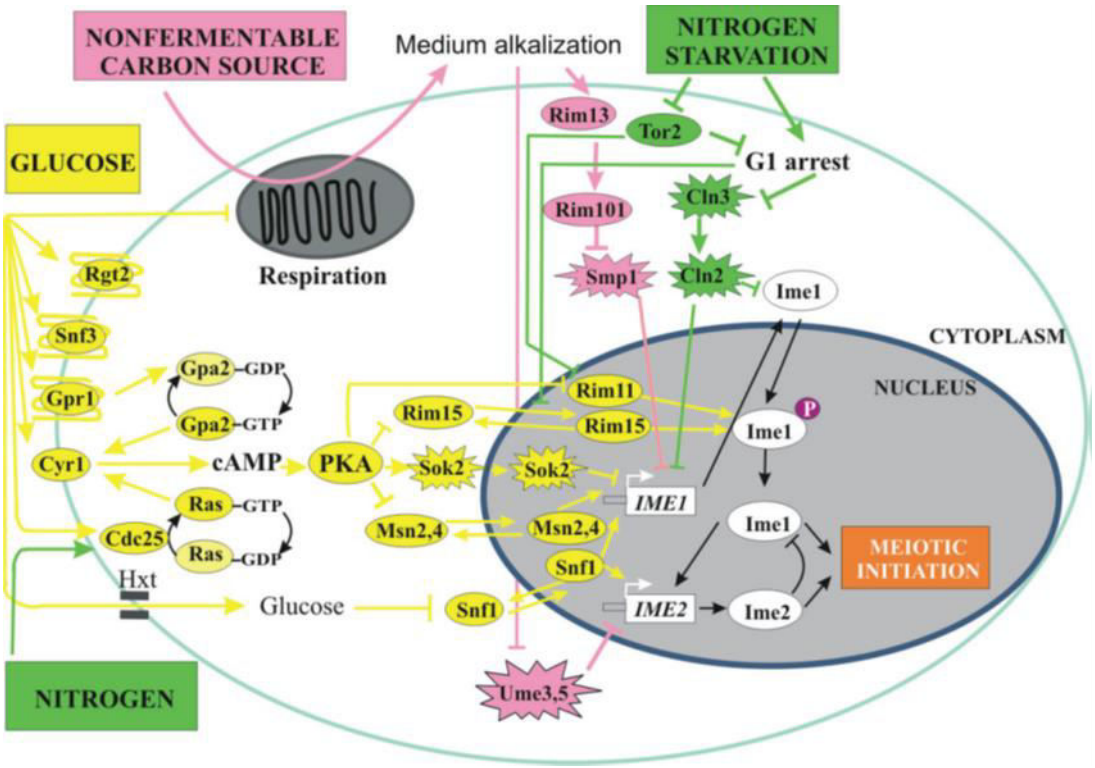

Figure 4: Sporulation is regulated by signal transduction pathways

IME1 is the responsible for the meiotic initiation and is regulated by the glucose repression pathway, the Rim101, the Tor2 and the Ras/cAMP/PKA pathway. Entry in meiosis is inhibited by glucose, which activates the repressor Sok2 and inactivates the activator Msn2/4 and Snf1. Furthermore, glucose does not permit the alkalization of the medium, important for the inhibition of the repressor Smp1. Other components, which regulate meiotic entry by phosphorylation of Ime1 are the kinases Rim11 and Rim15 and either the presence of glucose or the nitrogen inhibit their kinase activity. For permitting the expression of *IME1* and subsequently entry in the nucleus, cells have to arrest in G1 phase. Once expressed Ime1 transcription factor leads to the expression of *IME2* and the resulting Ime2 kinase activates the early genes responsible of the initiation of meiosis (Piekarska et al., 2010).

Several signal transduction pathways are involved in the initiation of meiosis. The Rim101 signaling pathway senses the alkalization of the medium due to CO_2 produced by the respiratory metabolism of a non-fermentable carbon source like potassium acetate (KOAc). The resulting CO_2 forms H_2CO_3 that then dissociates in ion bicarbonate HCO_3^- + H^+. This leads to the alkalization of the medium, which is important for the initiation of meiosis (Hayashi et al., 1998a; Ohkuni et al., 1998). The alkalization is ensured by the conversion of acetate into acetic acid which removes the ions H^+ from the medium (Dickinson et al., 1983, Mollapour and Piper, 2007). In presence of a high external pH the highly conserved pH sensus pathway Rim101 is activated. The Rim13 protease catalyzes the activation of Rim101 transcription factor by proteolytic cleavage (Lamb et al., 2001). Activated Rim101 represses Smp1, the repressor of *IME1* (Su and Mitchell, 1993). Rim101 represses also Nrg1, the transcriptional repressor of stress responsive genes like alkaline pH-induced genes (Lamb and

Mitchell 2003). Alkalization of the medium activates the Ume3/Ume5 complex, which is homologous to the cyclin-dependent kinase complex. Activity of Ume3/Ume5 is important for the induction of the transcription of *IME2,* a gene which encode for Ime2 kinase (Cooper and Strich, 2002; Ohkuni and Yamashita, 2000). Starvation for nitrogen is sensed by the target of rapamycin complex1 (TORC1) signaling pathway (Loewith and Hall, 2011). TORC1 is a complex formed by the Tor1 and Tor2 kinases. The activation of the pathway does not include directly the sporulation genes, but controls genes that are required for growth arrest (Hardwick et al. 1999). Tor2 is involved in the entry in meiosis. In presence of nutrients Tor2 leads cells to exit the G1 phase and entry in the S phase (Barbet et al., 1996; Kunz et al., 1993). In absence of nutrients Tor2 is inactive and cells arrest in the G1 phase of the cell cycle. This occurs because there is a drastic decrease of the cyclin Cln3 (Gallego et al., 1997; Parviz and Heideman, 1998). Cln3 inhibits *IME1* expression by activating the dependent kinase Cdc28 which promotes the transition from G1 to S phase. Therefore, if this cyclin is not present, entry in meiosis is promoted (Colomina 1999). There is also an indirect way by which entry in meiosis is favorite (Purnapatre et al., 2002). Cln3 can activate Swi4-Swi6 complex, which is required for the expression of the G1 cyclins *CLN1-CLN2* and drives the transition to S (MacKay et al., 2002). This happens in the G1 late phase of the cell cycle (Levine et al., 1996). The decrease of Cln3 in this phase reduces also Cln2 and Swi6, which are repressors of *IME1* (Wijien et al., 2002, Purnapatre et al., 2002). The glucose repression pathway regulates different processes in response to glucose (Figure 5). In presence of glucose the repression of genes responsible for the metabolism of alternative carbon sources occurs (Johnston and Carlson, 1992; Ronne, 1995), therefore glucose is metabolized as preferred source. The Snf1 serine threonine kinase is the central player in the main glucose repression pathway (Celenza and Carlson, 1984). It is a heterotrimeric complex which consists of the catalytic subunit Snf1, the regulatory subunit Snf4 and one of the three β-subunits Gal83, Sip1 or Sip2 (Celenza et al., 1989; Jiang and Carlson, 1997). Intracellular glucose inactivates Snf1 while in absence of glucose the subunit Snf4 binds and opens the complex activating it, so Snf1 is active in phosphorylated form. Phosphorylation is performed by the three upstream protein kinases Sak1, Tos3, and Elm1 (Hong et al., 2003; McCartney and Schmidt, 2001; Wilson et al., 1996). Upon glucose exhaustion, the active Snf1-Snf4 complex binds the Gal83 subunit entering in the nucleus where it activates by phosphorylation the transcription factors Sip4 and Cat8. These latter are the responsible of the expression of the genes called glucose repressed genes which are involved in the metabolism of alternative carbon sources. This is done by binding to so-called carbon source responsive elements

(CSREs) in the promoters of these genes (Randez-Gil et al., 1997, Vincent and Carlson, 1998). The complex Snf1-Snf4-Gal83 inactivates by phosphorylation the transcription factor Mig1 leading to its derepression. Mig1 indeed is a repressor of the genes responsible for the metabolism of alternative carbon sources when glucose is present (Carlson, 1999; Schuller, 2003; Vallier and Carlson, 1994). Depletion of glucose leads the beta subunit Sip1 to bind the complex Snf1-Snf4 as well and to locate at the vacuolar membrane. Sip1 is involved in the regulation of nitrogen metabolism and meiosis (Zhang et al., 2010). In glucose grown cells the maintenance of the cytosolic Sip1 localization is dependent on PKA activity (Hedbacker et al., 2004b).

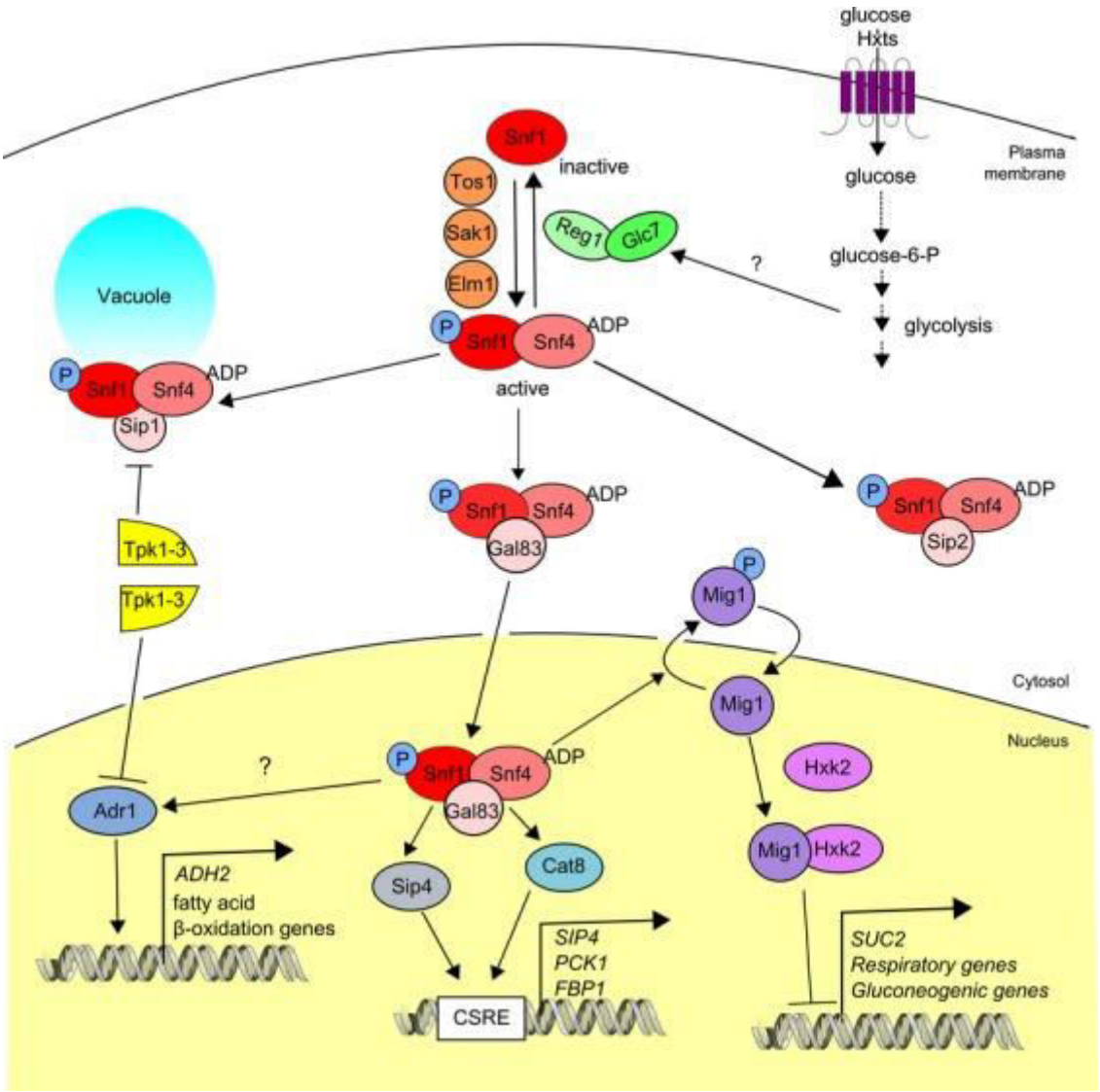

Figure 5: The Snf1 protein kinase as a central player in the main glucose repression pathway.

The Snf1 heterotrimeric complex; catalytic subunit Snf1, stimulatory subunit Snf4, and one of the three β-subunits: Gal83, Sip1, or Sip2. Sak1, Tos3, and Elm1 are kinases responsible of the phosphorylation of Snf1. Glc7 is a phosphatase in conjunction with its regulatory subunit Reg1

responsible for dephosphorylation of Snf1. According to the β-subunit to which the active form Snf1-Snf4 binds the complex acquires differential specificity for localization and target phosphorylation. Upon glucose exhaustion, the Snf1–Gal83 complex enters the nucleus and leads to the expression of genes involved in the use of alternative carbon sources. Adr1, Sip4, Cat8 and Mig1; transcription factors. (Conrad et al., 2014).

Snf1 has an interaction with Nrg1 (Vyas et al., 2001), which mediates glucose repression of multiple genes (Park et al., 1999; Zhou and Winston 2001; Lee 2013). The glucose induction pathway activates genes required for transport and metabolism of glucose (Kaniak et al., 2004; Palomino et al., 2005; Ronen and Botstein, 2006). It works through two membrane-spanning sensors Snf3 and Rgt2 each of which sense different amount of glucose. High concentrations of sugar are sensed by Rgt2 while either moderate or low glucose is sensed by Snf3 (Ozcan et al., 1996, 1998; Jiang et al., 1997). In presence of glucose the sensors generate a signal that stimulates degradation of Mth1 and Std1 by proteolysis (Flick et al., 2003; Spielewoy et al., 2004; Kim et al., 2006; Pasula et al., 2007). This occurs through activation of the kinase Yck1 (Moriya and Johnston, 2004). As result Rgt1 transcription factor, a repressor of the hexose transporters HXT is hyperphosphorylated and this leads to the inhibition of its DNA-binding activity (Polish et al. 2005). Furthermore, the release of Rgt1 from some HXT promoters requires cAMP-dependent protein kinase (PKA) activity (Gangedo et al. 2005). Rtg1 regulates initiation of meiosis in response to glucose by regulating the stability of Ime2 together with Snf3 (Gray et al., 2008). Progression of meiosis is ensured by another set of genes in which the global activator is called Ndt80 (Chu et al., 1998; Chu and Herskowitz, 1998; Pak and Segal, 2002b; Pierce et al., 2003). As with the initiation of meiosis, the transition to the meiotic division is also subjected to nutritional regulation (Figure 6). *NDT80* expression requires activation by Ime1/Ume6 and is therefore affected by nutritional control acting on Ime1 (Pak and Segal, 2002a). Furthermore, *NDT80* expression requires the kinase activity of Ime2 for the inhibition of the repressor Sum1.

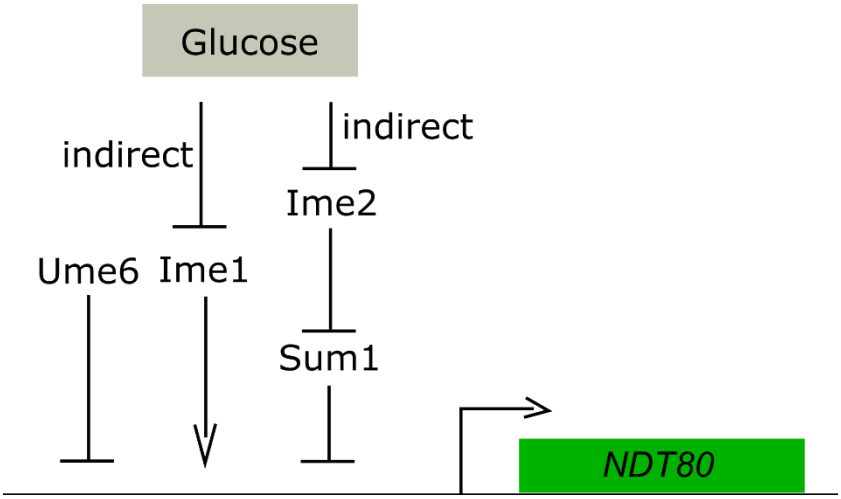

Figure 6: Entry into meiosis occurs in absence of glucose

Progression into the meiotic events is controlled by a transcription factor called Ndt80. Ime1 in presence of glucose cannot mediate the activation of *NDT80*. Ime2 is a kinase which mediates the repression of the repressor Sum1. Glucose inhibits Ime2. Glucose in an indirect way blocks the progression of the sporulation.

Ime2 as well is subjected to a nutritional regulation (Purnapatre et al., 2005; Gray et al., 2008). Indeed, glucose leads to the degradation of Ime2 and thus the block of the progression of meiosis by inactivating Ime2. Several ways are possible, one can be by inhibition of Snf1 (Honigberg and Lee, 1998) or through Grr1, a component of the SCF ubiquitin ligase (Purnapatre et al., 2005).

1.2.2.1 The Ras/cAMP/PKA pathway

The Ras/cAMP/PKA pathway affects metabolism, stress resistance, proliferation and sporulation in *S. cerevisiae* in response to nutrients (Thevelein and de Winde, 1999; Thevelein *et al.*, 2005). Cells deprived of food accumulate carbohydrates like trehalose and glycogen and if there is continuation of starvation the metabolism adapts to that condition by the expression of stress-resistance genes. Lack of nitrogen and presence of a fermentable carbon source leads to pseudohyphal growth (Pan et al., 2005). Yeast cells are able to adjust their transcriptional and metabolic profiles to select a developmental program for survival under the existing nutrient conditions. A lot is known on the regulation of the signal transduction pathways in presence of glucose. Two different circuits work in parallel to activate the protein kinase A (PKA), the Gpr1-Gpa2 circuit and the Ras-dependent pathway. The G-protein coupled receptor Gpr1 is a plasma membrane which senses the presence of external glucose (Lorenz et al. 2000; Rolland et al. 2000). It physically interacts with the alpha subunit of the

heterotrimeric G-protein (Gpa2) which is capable of binding guanine nucleotides. The interaction stimulates in Gpa2 the exchange of GDP for GTP which activates the adenylyl cyclase Cyr1 (Kraakman et al. 1999; Xue et al. 1998). Intracellular glucose activates a pathway in which Ras1 and Ras2 are involved. In presence of glucose these two small GTPases are present in an active GTP bound state. The responsible of the activation from the GDP inactive state are the GEFs, guanoside exchange factors Cdc25 and Sdc25. (Boy-Marcotte et al. 1996; Broek et al. 1987; Chrechet et al. 1990; Jones et al. 1991). The GTPase activating proteins GAPs instead are the responsible of the switch from the active to inactive form through the hydrolysis of bound GTP (Tanaka et al. 1989, Tanaka et al. 1990). In its active state Ras-GTP activates Cyr1. The glucose mediated signals by the two different circuits converge independently to Cyr1, an enzyme which produces cAMP from ATP when activated (Kataoka et al. 1985). Two phosphodiesterases Pde1 and Pde2, balance the levels of cAMP in the cell by degradation of this second messenger (Nikawa et al. 1987, Sass et al. 1986). PKA has three catalytic subunits called Tpk1, Tpk2 and Tpk3. The regulatory subunit is Bcy1 which binds the catalytic subunits and inactivates the kinases by forming a heterotrimeric complex (Toda et al. 1987). Precisely two regulatory subunits bind two catalytic subunits. When cAMP is present, it binds Bcy1 leading to its dissociation from the catalytic subunit. As result PKA is active (Figure 7).

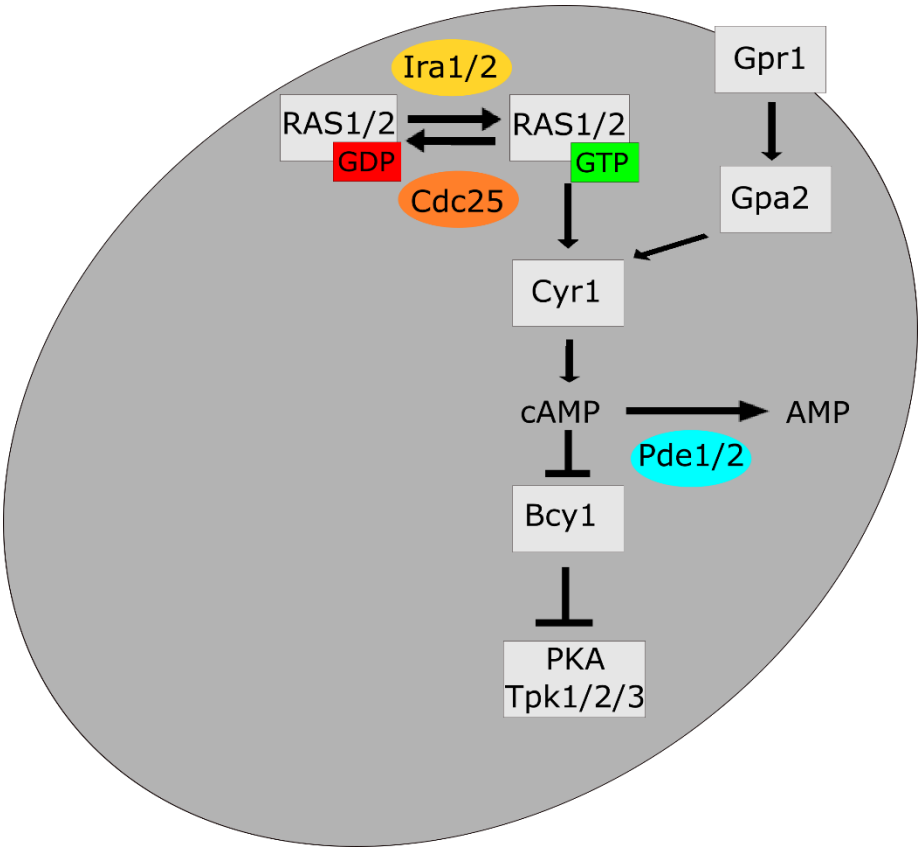

Figure 7: The Ras/cAMP/PKA pathway

In the presence of existing intracellular glucose the proteins Ras1 and Ras2 activate adenylyl cyclase Cyr1. Cdc25 regulates positively the activity of Ras1/2 while the activity is negatively affected by Ira1/2. The receptor system Gpr1/Gpa2 is responsible of the activation by extracellular glucose Activated Cyr1 produces cAMP and the overproduction of the second messenger is countered by the phosphodiesterases Pde1 and Pde2. cAMP binds the regulatory subunit of PKA called Bcy1 leading to its dissociation from the catalytic subunits of PKA called Tpk1, Tpk2, Tpk3. The catalytic subunit then is activated and target proteins are regulated by phosphorylation.

Previously it has been found a strong GFP-RBD signal in vegetative cells at the cell periphery and in the nucleus (Leadsham et al., 2009). The same nuclear and peripheral signal was found in vegetative cell and slightly reduced in sporulating cells (Jungbluth et al., 2012). Yet, during the same work, it was shown that acetate does not modulate Ras activity. Nevertheless, an increased high PKA activity by expression of the dominant active $RAS2^{G19V}$ variant led to a higher number of cells showing GFP-RBD in the cell periphery, indicating higher levels of Ras activity during sporulation. PKA has different target proteins involved in resistance to stress, carbohydrates metabolism, cell growth and meiosis, which regulates by phosphorylation (Ptacek et al., 2005; Ptacek and Snyder 2006). The three catalytic subunits Tpk1, Tpk2 and Tpk3 recognize different substrates and only 8 substrates are recognized by all three subunits, which indicates a high substrate specificity (Galello et al., 2010). Examples of PKA-dependent regulation targets are the transcription factors Msn2-Msn4 that are involved in the general stress response (Martinez-Pastor et al., 1996; Schmitt and McEnte, 1996). *MSN2/4* encode for two Cys_2His_2 Zink finger DNA-binding proteins that induce transcription of a certain number of environmental stress response genes involved in oxidative and osmotic stress, heat shock or lack of glucose (Causton et al., 2001; Gasch et al., 2000). In presence of glucose PKA phosphorylates the nuclear localization sequence of Msn2/4. As consequence, they localize in the cytoplasm. In absence of glucose PKA activity is diminished and the two transcription factors localize in the nucleus where they can play their role of activators of *IME1*. Indeed, they act on the genes which contain stress response elements in their promoters. Nuclear export of Msn2/4 depends also on other factors like Msn5, an export receptor which interacts with the small GTPase binding nuclear protein Ran. When this exportin is absent Msn2 accumulates in the nucleus (Alepuz et al., 1998; Garner et al., 2002). Nuclear localization of Msn2 is also controlled by stresses that modify the nuclear export signal (NES) of the protein (Gorner et al., 2002). Furthermore Msn2/4 are retained in the cytoplasm by Bmh2 (Beck and Hall 1999). In addition, Msn2 and Msn4 also directly induce the expression of *IME1* (Sagee et al., 1998).

1.2.2.2 PKA regulates entry in meiosis

The activity of PKA has an important role on the switch from mitosis to meiosis. High PKA activity is connected to vegetative growth while low activity leads diploid cells to enter meiosis. Exactly the Ras/cAMP/PKA pathway regulates entry in meiosis by inhibition of the expression of *IME1* which encodes for the transcription factor Ime1 (Matsumoto et al., 1982; Matsuura et al., 1990). High PKA phosphorylates Sok2 leading to its nuclear localization. Sok2 is a repressor which inhibits the transcription of *IME1* through binding the IREu of *IME1* (Shenhar and Kassir, 2001). Yet, PKA regulates initiation of meiosis by repressing Rim11 and Rim15, two kinases that through phosphorylation of Ume6 DNA binding protein induce the expression of *IME1* and *IME2* (Swinnen et al., 2006, Rubin-Bejerano et al., 2004).

1.2.2.3 PKA affects later phases of meiosis during the course of the sporulative process.

PKA affects meiotic divisions and spore formation. The transcription of two of the middle genes which define the onset of the meiotic divisions is regulated by PKA. Indeed, PKA regulates the Hap complex which is responsible of the expression of the middle gene *MPC70* (Lee et al., 2002) and affects the expression of *SPO74* (Wang et al., 2004). Studies have shown that cAMP levels rise during meiosis I and meiosis II and decrease toward the end of the spore formation (Uno et al., 1985) suggesting that PKA is still active after the entry in meiosis. Nutrients are sensed by yeast cells during the entire sporulative process but before and after the point of commitment cells react in a different way. Recently it has been shown that the Ras/cAMP/PKA pathway regulates spore formation after commitment to meiosis in a period between the beginning of meiosis I and the formation of meiotic plaques in meiosis II and is required for the regulation of spore numbers by acetate availability (Jungbluth et al., 2012). To do that the authors used a method to deplete specifically proteins in meiosis (Taxis et al., 2009; Jungbluth et al., 2010). Depletion of Ras1 and Ras2 proteins (Ras) or depletion of the subunit Tpk2 (Tpk) in a strain in which Tpk1 and Tpk3 subunits were deleted, leads to an increase of the spore formation at low concentrations of potassium acetate. The hyperactivation of PKA by depletion of the regulatory subunit Bcy1 after entry in meiosis leads to a decrease of the spore formation. Bcy1 is destabilized by a tev protease that is produced under the control of the Ime2 promoter. Therefore, cells have to enter meiosis to induce downregulation of Bcy1 which results in activation of PKA. During the same work, high PKA activity was induced by hyperactivation of $RAS2^{G19V}$ allele which in vegetative cells blocks meiosis almost completely (Matsuura et al., 1990), therefore the assay has been

performed using the *SPS1* promoter which is induced after commitment to meiosis (Chu et al., 1998; Chu and Erskowitz, 1998; Primig et al., 2000). Cells sporulated but the number of spores decreased significantly. Production of cAMP by adenylyl cyclase Cyr1, a component of the Ras/cAMP/PKA pathway, activates PKA. Modification of the adenylyl cyclase Cyr1, which then has a reduced activity with the consequent reduction of the activity of PKA conducts to an increase of the spore formation at low concentrations of nutrients similar to strains that are depleted of Ras or Tpk (Jungbluth et al. 2012). The Ras/cAMP/PKA pathway regulates carbohydrate metabolism and influences the mitochondrial respiratory capacity. The pathway exerts during vegetative growth and during sporulation a negative impact on the glyoxylate cycle and on the TCA cycle affecting the amount and the activity of certain enzymes (Hedbecker et al. 2004; Ordiz et al. 1996; Ptacek et al. 2005, Roosen et al. 2005; Schneper et al. 2004; Swieger et al. 2006; Zaman et al. 2008; Jungbluth et al. 2012). The deletion of genes of the glyoxylate encoding for the enzymes which catalyze the conversion isocitrate to malate (*ICL1*) and glyoxylate to malate (*MLS1*) affected spore formation by increasing the production of dyads (Nickas et al. 2005). The authors postulated that an intermediate metabolite of the glyoxylate cycle is important for a production of a high number of spores. Mls1 is phosphorylated by Tpk3 *in vitro* (Ptacek et al., 2005) therefore PKA might regulate spore numbers control by affecting the glyoxylate cycle. It has been suggested that another metabolite which accumulates during the metabolism of acetate like CO_2 is also important for the regulation of the spore numbers (Jungbluth et al. 2012). Tpk3 phosphorylates Ady1 *in vitro* (Ptacek et al., 2005). Ady1 is a protein active in meiosis II which is required for the localization of all the essential MP components to the SPBs and has a control role in the activity of the MP (Deng and Saunders, 2001; Jungbluth et al., 2012), therefore it could represent a link between the PKA pathway and the spore formation. PKA is embroiled in spore biogenesis because it regulates Smk1 MAP kinase responsible of the synthesis of mature spore wall. Two *smk1-2* mutants lead to a defect in spore morphogenesis (Wagner et al, 1999) and two mutants in the Ras pathway, which decrease the PKA activity can suppress such defect. The mutants are partial function alleles of two components of the pathway, *ess2* is the allele of *CDC25* which encodes the GDP/GTP exchange factor (GEF) for Ras1 and Ras2, *ess67* is the allele of *CYR1* which encodes adenylyl cyclase. Consistent with the known role of glucose in Ras/cAMP/PKA signal transduction pathway the effect of the mutations on the *smk1-2* phenotype required the presence of low levels of glucose. In the same article, it has been shown that Ime2 kinase is responsible of the activation of Smk1 in three different ways. Ime2 can regulate positively Ndt80 which triggers either components of

the Smk1 pathway or can activate Cak1, a kinase which is known phosphorylates Smk1. Ndt80 transcription factor can also activate Smk1 directly. Overall the data suggest that Ras/cAMP/PKA pathway could control Smk1 pathway by inhibiting Ime2 (Mcdonald et al., 2009).

1.2.2.4 Carbon dioxide changes spore formation

PKA regulates carbohydrate metabolism, it affects the capacity of ATP in the mitochondrial respiratory and the enzymes of the TCA cycle in vegetative cells (Hedbacker et al., 2004; Leadsham and Gourlay 2010; Ordiz et al., 1996; Ptacek et al., 2005; Roosen et al., 2005; Schneper et al., 2004; Swiegers et al., 2006; Zaman et al., 2008). It seems that it could influence spore formation by metabolic regulation. Previous studies showed that carbon dioxide is an agent which rises sporulation by alkalizing the medium (Hayashi et al., 1998a; Hayashi et al., 1998b; Ohkuni et al., 1998). It is also a regulator of a specific group of fungal adenylyl cyclases (Bahn et al., 2005; Klengel et al., 2005; Mogensen et al., 2006). Cyr1 from *S. cerevisiae* belongs to class III of the adenylyl cyclases and as many members of this class it is bicarbonate-sensitive (Cann et al., 2003). The responsible of the bicarbonate responsiveness is a lysine in the catalytic center (Cann et al., 2003; Hall et al., 2010) and in principle the bicarbonation causes a conformational change in the active center which leads to a higher processivity of the enzyme (Steegborn et al., 2005). Recently it was found out that also an increase of the intracellular bicarbonate enhances spore formation (Jungbluth et al., 2012). In this work, it was shown that the acetate metabolite of the TCA cycle carbon dioxide then converted in bicarbonate, acts upstream of the MP formation but the authors could not find evidence that it does affect Cyr1 and PKA.

1.2.2.5 Targets of PKA involved in other processes

PKA is involved in the biogenesis of ribosomes (Martin et al., 2004). It controls the localization of Sfp1. Sfp1 is a transcription factor which regulates the ribosomal proteins (RP) gene expression by binding their promoter when it is localized to the nucleus (Marion et al., 2004). PKA also represses the activity of Crf1, the repressor of the ribosomal proteins RP by inhibition of the kinase Yak1 (Martin et al., 2004). Moreover, PKA regulates growth by controlling the expression of ribosomal biogenesis genes. As matter of fact, active PKA phosphorylates Maf1, the repressor of genes like 5sRNA and tRNA which are transcriptionally regulated by RNA polymerase III. The phosphorylation leads to the

cytoplasmic localization of the repressor Maf1 (Moir et at., 2006; Willis and Moir, 2007). Yet, transcription of the rDNA by the polymerase I RNA is affected by PKA by the Rrn3 protein (Howard et al., 2003). Another role of PKA is to repress Tod6 and Dot6, two repressors of the ribi genes (Lippmann and Broach, 2009). PKA is connected to other signaling pathways. For example, active PKA affects negatively the Snf1 pathway by phosphorylating Sak1, a kinase which activates Snf1 (Barret et al., 2012). PKA as well phosphorylates and regulates the β subunit Sip1 preventing the localization of the Snf1 complex at the vacuolar membrane (Hedbecker et al., 2004). Furthermore, PKA shares with Snf1 downstream targets like Adr1 and Msn2 (Cherry et al., 1989; Ratnakumar et al., 2009; Gorner et al., 1998; Gorner et al., 2002, De Wever et al., 2005). Snf1 kinase is responsible of the initiation of meiosis by expression of *IME* and *IME2* (Honigberg and Lee, 1988). Itas been shown that lowered PKA activity which induces nucleus localization of Msn2/Msn4 transcription factors stimulates the expression of *PNC1* (Ha et al., 2014). Binding sites for the stress-responsive zinc-finger transcription factors Msn2 and Msn4 have been identified in the *PNC1* promoter (Ghislain et al. 2002). During calorie restriction (CR), Msn2/4 are present in the nucleus where can express *PNC1* (Medvedik et at., 2007) and under glucose restriction Pnc1 levels are highly induced (Anderson et al. 2003).

1.2.3 Transcriptional regulation of sporulation

The phases of sporulation are controlled by a regulatory protein cascade, which is induced by genetic and environmental signals (Figure 8). Several transcripts in *S. cerevisiae* are related to sporulation events and according to the particular sporulation stage to which they belong, genes are grouped in early, middle, mid-late and late genes (Chu et al., 1998; Primig et al., 2000). Early genes are responsible for DNA replication and recombination which occur during prophase. Meiotic divisions are activated by middle genes while morphogenesis of the spores is under the control of late genes. The correct order of the activation of all genes is ensured by the co-expression of the same transcription factors, which control all the genes of the groups. That is possible because all members of each class of sporulation genes have common regulatory sequences within their promoters.

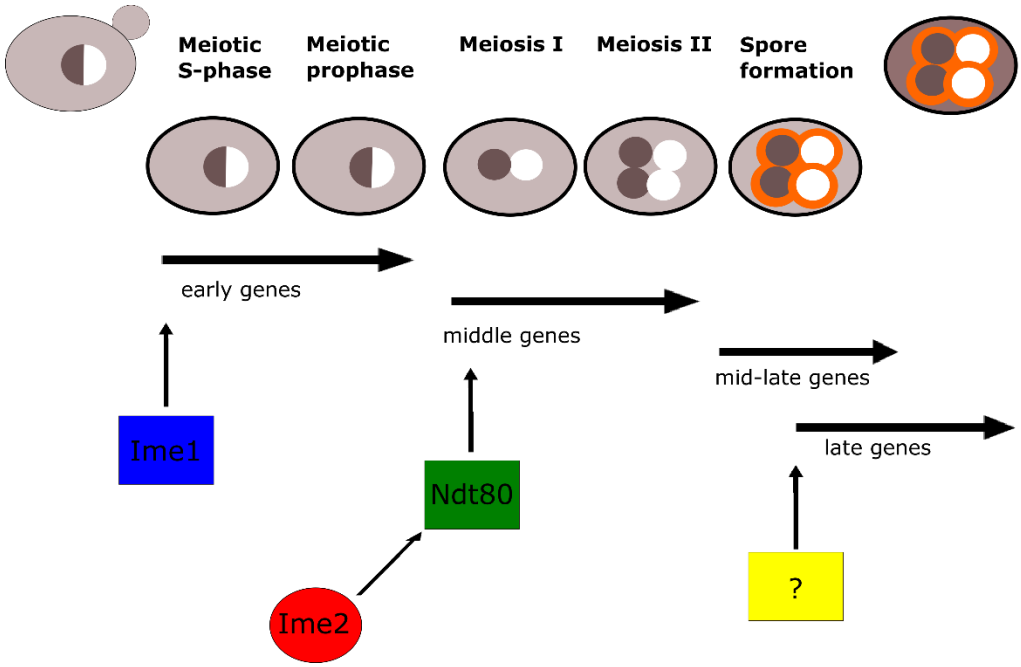

Figure 8: Transcriptional regulation during meiosis

Three categories of genes are responsible of the entire meiotic process. According to the different meiotic phases they are distinguished in early, middle and late genes. Ime1 transcription factor induces the early genes. Ime2 kinase leads to the expression of *NDT80*, which encodes a transcription factor that regulates the middle genes. The responsible of the activation of late genes is unknown.

The activators of early and middle genes are respectively Ime1 and Ndt80 transcription factors (Granot et al., 1989: Kassir et al., 1988; Matsuura et al., 1990; Mitchell et al., 1990; Chu and Erskowitz, 1998; Pak and Segall, 2002 a, b; Pierce et al., 2003) while the factor required for the activation of late genes remains unknown. Early genes are inhibited by other two co-repressor complexes, Ume6 and Sin3 form a complex with Rpd3 deacetylase which lead to the deacetylation of chromatin and to the repression of the transcription by blocking the functioning of the activator proteins (Vershon and Pierce, 2000; Kadosh and Struhl, 1998). In the other complex the ATP-dependent chromatin-remodeling factor Isw2 which is recruited by Ume6 represses the early meiotic genes during mitotic growth (Goldmark et al., 2000; Fazzio et al., 2001). Ume6 is a DNA binding protein, which binds during vegetative growth in the URS1 sequence (upstream repression sequence) of the promoter of the early genes (Anderson et al., 1995; Steber and Esposito, 1995; Strich et al., 1994). Middle genes are repressed by Sum1 which binds to the specific site called MSE (middle sporulation elements) in the promoter of middle genes (Xie et al., 1999). Sum1 prevents the transcription by recruiting Hst1 through the interaction with Rfm1 (McCord et al., 2003; Weber et al 2008; Xie et al., 1999.) Not a lot is known about the repression of late genes (Figure 7). One

complex involved is Ssn6-Tup1 which is also required for the repression of many other sporulation genes (Friesen et al., 1997; Mizuno et al., 1998; Zhang and Reese, 2004).

1.2.3.1 Regulation of the genes which control the event of meiosis and sporulation

When the major initiator of meiosis *IME1* is expressed meiosis can initiate. The promoter of *IME1* is divided into four upstream controlling sequences UCS (Sagèe et al., 1998), which affect its transcription. Ime1 transcription factor induces the expression of early genes included *IME2*, a gene which encodes for Ime2 kinase. Ime2 promotes the transition G1-S by reducing the levels of Sic1 an inhibitor of the complex Cdc28/Clb5,6 (Benjamin et al., 2003). Ime2 phosphorylates and activates during meiosis the middle gene *NDT80* promoting then the transition from the S to M phase (Benjamin et al., 2003; Sopko et al., 2002). Ime2 is required to shut off Ime1 after prolonged starvation. (Smith and Mitchell, 1989) and Ime1 is also inactivated after transition from prophase to metaphase. Ime2 phosphorylates Ime1 inducing its degradation by proteasome and therefore leading to the stop of the expression of the early genes (Mitchell 1994; Gutmann-Raviv et al., 2002). Furthermore, it was shown that Ime2 also plays a role in the last stage of sporulation, spore morphogenesis. Ime2 exerts a positive influence on the activity of Smk1, a MAP kinase required for the formation of the spore wall (Huang et al., 2005; Krisak et al., 1994). The transcription factor Ndt80 induces the middle genes and promotes the passage from prophase I to metaphase I by a positive autoregulatory loop (Winter, 2012). Moreover, Ndt80 positively regulates its own expression (Pak and Segall, 2002a, b). The late meiotic genes are classified into mid-late and late genes depending on the time of their activation (Chu et al., 1998; Vershon and Pierce, 2000). Among the middle-late genes there are those genes that are involved in the formation of spores, while the late genes are involved in their maturation (Kassir et al., 2003; Mitchell, 1994). Nothing is known about the transcription factor binding in the promoter of the late meiotic genes. Nevertheless, both set of genes need two components of the sporulation specific MAPK cascade to be transcribed. These are Smk1 and Sps1 (McDonald et al., 2009; Iwamoto et al., 2005).

1.2.4 Regulation of the number of spores

The regulation of spore numbers is called spore number control (SNC) and occurs to the spindle pole bodies SPBs. It is is that regulate the amount of meiotic plaque protein which are essential for the MP formation. An adequate supply of nutrient like potassium acetate (KOAc) during sporulation leads to the formation of four spores within an ascus (tetrade). It has been observed that with reduced amounts of this fermentable carbon source the levels of the MP

components are reduced. As consequence, the number of spores within an ascus is decreased as well while the number of cells, which have carried out meiotic divisions but have not formed spores, is increased (Bajgier et al., 2001; Gordon et al., 2006; Nickas et al., 2003; Taxis et al., 2005; Wesp et al., 2001). SNC is important for the population of yeast cells because is beneficent for the fitness of the population of postmeiotic cells. (Taxis et al., 2005). The modification of the SPB with the MP depends on the age of the respective SPBs. The first SPB is formed during the last cell division while the second SPB is formed before meiosis I. Prior meiosis II the last two SPBs are formed. In a dyad for example the two MPs are formed to the newer SPBs (Nickas et al., 2004). Younger SPBs are preferred to be modified with the MP due to the Nud1 component which provokes differences between the SPBs. This is important for the regulation of the genome inheritance. Each SPB forms a polar center at the end of the meiotic spindle. Such differences in the inheritance of the SPBs as a function of age leads to the formation of two polar axes along the meiotic spindles. This polar situation is called spindle polarity (Taxis et al., 2005; Gordon et al., 2006). Therefore, spindle polarity plus the MP components abundance are important for the final number of spores in the ascus. According to the nutritional conditions the SNC maximizes the number of gametes per cell in a way that all the formed spores are viable. Furthermore, SNC improves mating between spores having opposite mating type in the ascus (Taxis et al., 2005). The two spores in a dyad have non-sister chromatids because their genomes derive from different meiosis II spindles (non- sister genomes). Crossings between spores in the same ascus have been already observed for yeasts (Winge and Laustsen, 1937). The interbreed between non-sister spores ensures heterozygosity in the offspring and guarantees genetic diversity within the population (Taxis et al., 2005). How exactly the nutrient availability affects the amount of MP proteins or the differences between the spindle poles has not been investigated so far. Spore number control is affected by different components including two proteins. One of the proteins is Ady1 (accumulation of dyads 1) which has a role in the formation of the MP, it localizes all the MP components to the SPB (Deng and Saunders, 2001; Jungbluth et al., 2012). It has a function in the synthesis of the prospore membranes, in fact, it was shown that in its absence there is a dislocation of several proteins like the lipase Spo14 required for the prospore membrane formation, or Don1, a protein of the SPB required for the prospore membrane growth and closure. That protein is localized like a ring to the leading edge of the prospore membranes (Deng and Saunders., 2001). Ady1 was found localized just to the nucleus of mononucleate meiotic cells and in absence of it dyads are predominantly formed (Deng and Saunders, 2001). Ady1 is phosphorylated by PKA *in vitro* (Ptacek et al., 2005) and it has been

shown that it has a genetic interaction with the MP components Mpc54, Mpc70 and Spo74 (Jungbluth et al., 2012). So Ady1 could regulate the MP in a PKA-dependent manner. The other protein is Ady2 (accumulation of dyads 2), an acetate transporter which uptakes acetate in the cell (Paiva et al., 2004) and regulates the amount of MP proteins (Taxis et al., 2005). Ady2 has a functional relation with the adenylyl cyclase Cyr1 (Jungbluth et al. 2012). Strains in which Ady2 is missing have a lower capacity to metabolize acetate. It was shown that the reduction of the activity of Cyr1 in a *ady2*Δ mutant increased the alkalization of the medium and therefore the acetate metabolism defect. The two knock out strains *ady1*Δ and *ady2*Δ lead to the accumulation of dyads (Rabitsch et al., 2001). Dyads are formed also when components of the glyoxylate cycle required for the conversion from acetate to glucose are deleted (Nickas et al., 2004). In such study, the authors found out that the glyoxylate pathway regulates the modification of mother SPBs via recruitment of Mpc70 and Spo74 during sporulation. This implies a role of the acetate metabolism in the regulation of spore numbers.

1.2.5 Acetate metabolism during sporulation

Acetate could enter in the cell through the acetate transporter Ady2 (Pavia et al., 2004) via a proton symport mechanism (Casal et al., 1996; Makuc et al., 2001). Another way to reach the inner of the cell is through passive diffusion over the plasma membrane channel aquaglyceroporin Fps1 in the form of acetic acid (CH_3COOH) (Dickinson et al., 1983; Mollapour and Piper, 2007). Acetate joins the coenzyme A and the resulting acetyl-CoA is used either in the catabolic or anabolic metabolism. The acetyl-CoA enters in the glyoxylate cycle which shares enzymes of the TCA and the resulting succinate is utilized in the TCA within the mitochondria (Kornberg, 1996; Lorenz and Fink, 2001). To enter inside the mitochondria the acetyl-CoA needs a carrier molecule like the carnitine shuttle. Once inside the mitochondria the acetyl-CoA-carnitine molecule is subjected to cleavage and finally acetyl-CoA can be used by the TCA cycle (Bremer, 1983; Strijbis and Distel, 2010). Here it is catabolized in CO_2 and H_2O and the gained electrons will be necessary for the production of ATP. Beside that the acetyl-CoA through glyoxylate cycle leads to the formation of oxalacetate as well which then is used in the gluconeogenesis to form glucose (Kondrashov et al., 2006; Lorenz and Fink, 2002).

1.3 The NAD salvage pathway

The main role of the nicotinamide adenine dinucleotide NAD in metabolism is to transfer electrons from one molecule to another. It is involved then in redox reactions and is an

essential cofactor used in many biological processes like longevity, life span extension and transcriptional regulation (Lin and Guarente, 2003). NAD is regenerated in one of the major NAD biosynthesis pathways called NAD salvage pathway. Such pathway is organized as recycling mechanism in which each intermediate is a precursor for the next intermediate, and where NAD+ levels can be maintained by regeneration of NAD+ from nicotinamide. In this pathway, nicotinamide is converted first in niacine (nicotinic acid) in presence of water by a nicotinamidase Pnc1 and then in nicotinic acid mononucleotide by a transferase. This is the point of convergence with the other pathway called de novo pathway (Lin and Guarente, 2003), therefore the two steps of the pathway which require the use of a transferase and a synthase are shared by the two pathways. The conversion from NAD to nicotinamide in the pathway requires Sir2 histone deacetylase. The nicotinamidase Pnc1 is an activator of Sir2, it prevents the accumulation of nicotinamide that is an inhibitor of Sir2 (Gallo et al., 2004).

1.4 Ribosome biogenesis

Ribosome biogenesis is a very tightly regulated process, and it is closely linked to other cellular activities like growth and division (Thomson et al., 2013). Ribosomes are the formation sites of the proteins and consist of two subunits, the small one and the large one. Both subunits are composed by ribosomal RNA (rRNA) and ribosomal proteins. Ribosomal proteins are synthesized by ribosomal genes (RP genes) while the rRNA is synthesized by ribosomal DNA (rDNA). Ribosomal proteins are formed in the cytoplasm and imported into the nucleus while rRNA is synthetized and processed in the nucleolus. Ribosomal subunits then are built inside the nucleus and subsequently transported into the cytoplasm (van Riggelen et al., 2010). Another set of genes which encode for non-ribosomal proteins are called ribi genes which are involved in ribosome synthesis and maturation (Gasch et al. 2000; Wade et al. 2001; Jorgensen et al. 2002). The function of the Ribi genes then is to boost translational capacity. Such proteins assemble and modify rRNA and RPs in the nucleolus (Hughes et al. 2000; Wade et al. 2001; Fatica and Tollervey 2002; Jorgensen et al. 2002). The regulation of ribosome biogenesis occurs at a transcriptional level and is controlled by three different RNA polymerases. RNA polymerase I transcribes a rDNA which encodes for 35S rRNA, RNA polymerase II transcribes the ribosome protein genes RP and the RNA polymerase III produces 5S rRNA and tRNA (Martin et al. 2006). In *Saccharomyces cerevisiae*, a single repeat unit of the rRNA gene cluster (rDNA) consists of two transcribed genes (5S and 35S RNA genes) and two non-transcribed regions *NTS1* and *NTS2* in which the origin of replication (ARS) and the replication fork barrier (RFB) are respectively located.

RFB is exactly located near the end of the 35S rRNA gene. In the S phase of the cell cycle replication starts at the ARS in the two directions. The RFB allows progression of the replicative fork in the direction of 35S rRNA transcription but not in the opposite direction. Indeed, a role of the RFB is to be a barrier to prevent collision between replication and transcription machineries (Kobayashi and Horiuchi 1996). A nucleolar protein required for the activity of the RFB is Fob1, it binds and blocks the RFB (Kobayashi 2003). The molecular mechanism by which Fob1 mediates these activities has not determined yet but is known that Fob1 has a zinc finger motive with which it binds the DNA (Kobayashi 2003).

1.5 Nrg1 and Yap1 transcription factors

Nrg1 is a transcriptional repressor (Park et al., 1999; Vyas et al., 2001). Nrg1 protein levels are elevated in response to glucose limitation or growth in nonfermentable carbon sources (Berkey et al., 2004). Nrg1 regulates the stress responsive genes by binding the stress response elements (STREs) present in their promoter (Vyas et al., 2005). An example of such genes are the alkaline pH-induced genes. Nrg1 then functions as an inhibitor of alkaline pH responses by repressing alkaline pH-induced genes (Lamb and Mitchell 2003). In presence of an alkaline pH the transcription factor Rim101 represses *NRG1* (Lamb and Mitchell 2003). As consequence stress genes can be expressed. Moreover, Nrg1 together with Rim101 bind simultaneously to adjacent target sites within the 42-bp negative regulatory element (NRE) present in the *DIT1-DIT2* intergenic region *in vitro* and act as corepressors of these sporulation-specific genes *in vivo* (Rothfels et al., 2005). Nrg1 as well plays a role in the glucose repression of several glucose-repressed genes (Park et al., 1999; Zhou and Winston 2001; Lee 2013). Indeed, it interacts with Snf1 protein kinase (Vyas et al., 2001) which is the main component of the glucose repressed pathway (Celenza and Carlson, 1984). The Snf1 kinase is activated in response to glucose limitation and relieves Nrg1-mediated repression of the glucose repressed genes. Yap1, a member of the AP-1 family of transcription factors, activates the transcription of anti-oxidant genes in response to oxidative stress (Toone and Jones 1999). The transcriptional activity of Yap1 is regulated by its cellular localization. The N-terminal region contains a nuclear localization signal (NLS) while the C-terminal region contains a nuclear export signal (NES) (Kuge et al. 1997). In absence of oxidative stress Yap1 is exported from the nucleus via the exportin Crm1 (Kuge et al. 1998). However, in presence of hydrogen peroxide, Hyr1, a protein similar to glutathione peroxidase, catalyzes the formation of an intramolecular disulfide bond in Yap1 (Kuge et al. 2001). This conformational change allows Yap1 to accumulate in the nucleus (Gulshan et al. 2005). Yap1

accumulates in the nucleus also in response to carbon stress but it does not depend on Snf1, PKA, TOR or Yak1 signaling pathways (Wiatrowski and Carlson, 2003). The only connection between Yap1 and Snf1 is the two yeasts hybrid interaction between Yap1 and Sip2, a subunit of Snf1 kinase (Wiatrowski and Carlson, 2003).

1.6 Tools to study the regulation of spore formation

1.6.1 The TIPI system

Reverse genetics is used to study the function of a gene and the corresponding gene product. The function of a specific gene can be studied by analyzing the phenotypic effects of specific engineered gene sequences, but if a gene is fundamental for vegetative growth then problems could incur. The possibility to modify a target protein with a signal which confers protein instability could solve these issues. This method widely used in cell biology can employ tools called conditional degrons which can manipulate *in vivo* protein abundance or activity. Degrons are sequences which upon a particular signal like heat or small molecules induce protein degradation (Dohmen et al., 1994). The degradation occurs by the ubiquitin-proteasome system (UPS) (Taxis et al., 2009). The signal used to induce degradation is a so called N-degron which comprises a destabilizing N-terminal residue and one or more internal lysine residue accessible to an ubiquitin-protein-ligase (Varshavsky, 2011). The tobacco etch virus (TEV) protease induced instability system (TIPI; Taxis et al. 2009; Jungbluth et al. 2010) is used to control protein stability. This method uses a tag fused to a target protein and contains one or two distinct degrons. The activation of the degron occurs due to cleavage by TEV protease pTEV (named also pTEV+ protease). The degron is formed by an unstructured domain cODC1 derived from the murine ornithine decarboxylase (ODC) C-terminus (Jungbluth et al., 2010). The sequence contains the motif cysteine-alanine (CA) and mediates the destabilization activity due to its proteasomal association. Other elements important for destabilization is an unstructured region flanking the CA motif (Takeuchi 2007; Takeuchi 2008). This is followed by the TEV protease recognition site (ENLYFQ-F), the N-degron with the destabilizing amino acid phenylalanine (F) and completed by an accessible lysine. Finally fused to the degron there is an affinity domain $SF3b^{155381\text{-}424}$ to enhance the proteolytic activity of the TEV protease via interaction with the spliceosome subunit 14 (Spadaccini et al., 2006) (Figure 9 A). The expression of the *pTEV+ protease* is controlled by an appropriate promoter which can be inducible or active during a specific cell-cycle or developmental process. The binding of the pTEV+ to the recognition site leads to the cleavage of the tag and

to the deprotection of the dormant degron resulting in rapid proteasomal proteolysis of the target protein (Figure 9 B)

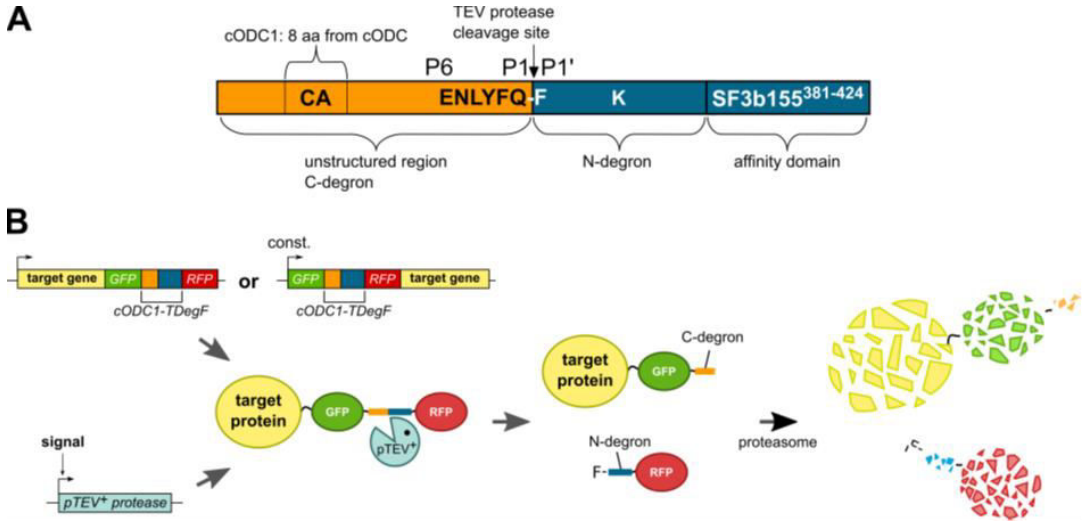

Figure 9: Regulation of target protein abundance by the TEV protease induced protein instability (TIPI) system

A) Structure of the bidirectional TIPI tag. The bidirectional degron consists of a C-degron separated from a N degron by the TEV protease recognition site ENLYFQF. The C-degron possess an unstructured region with cODC1 while the N-degron is fused to the affinity domain $SF3b^{381-424}$. This latter binds to the human protein p14 which is fused to the TEV protease. **B)** Function of the TIPI system. It is possible to attach the TIPI tag to the 3'-end or to the 5'-end of the target gene. When the TEV protease is induced by a specific signal the degron is active and induces proteasomal proteolysis of the target gene (Christian Renicke doctorate thesis 2016).

The TIPI system was used to study the role of genes in meiosis where the $pTEV^{+}$ expression was controlled by the promoter of the early-meiotic gene *IME2* (Jungbluth et al. 2012). However, the lower expression levels of the meiosis-specific *IME2* promoter required the introduction of several gene copies of the construct on high copy plasmid, therefore the system has been improved by exchanging the constitutive promoters (*ADH1* and *CYC1*) previously used with the *MCD1* promoter (also called *SCC1* promoter), a gene which encodes a mitosis-specific subunit of the cohesion complex. *MCD1* is not expressed in meiosis. The resulting tag is called sid-tag.

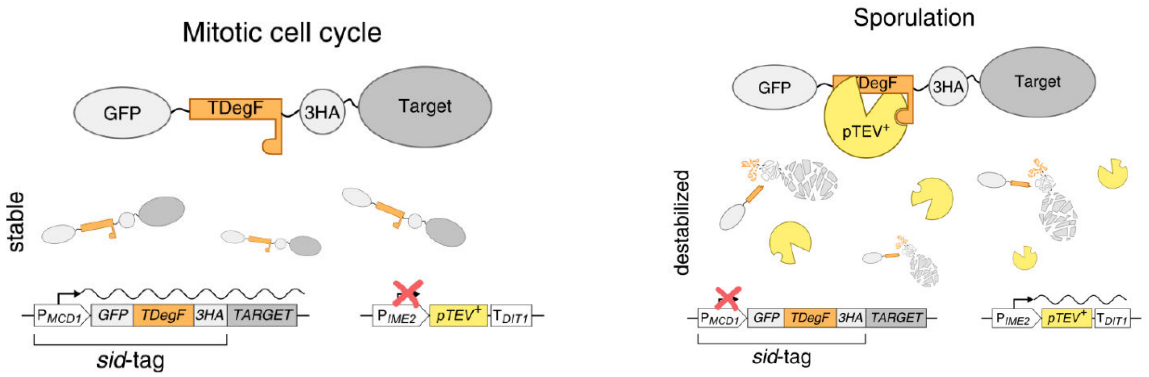

Figure 10: Enhanced sporulation-induced protein depletion

Sporulation-induced protein depletion (sid). The sid-tag is composed by the MCD1 promoter and the TDegF flanked by GFP and 3HA-tag. This tag is fused to the 5'-end of the target gene. The *pTEV*+ gene with the *IME2* promoter and the *DIT1* terminator is present at one or more chromosomal loci. The passage from the mitotic cell cycle to the sporulation leads to the downregulation of the promoter *MCD1* and pTEV+ is generated after the starting of meiosis. The TDegF is then is cleaved by the TEV protease, the N-degron is activated and the target is degraded (Renicke et al., 2017).

Moreover, the *CYC1* terminator has been replaced by the more efficient *DIT1* terminator which is more active under starving conditions (Yamanishi et al. 2013; Ito et al. 2013). The resulting construct has been chromosomally integrated at two different loci (*TRP1, HIS3*) to further increase the *pTEV* expression (Figure 10) (Renicke et al., 2017).

1.6.2 Light regulated adenylyl cyclase bPAC

Optogenetics is a biological technique that involves the usage of light to control cells which have been genetically modified. Mostly in neuroscience a combination of techniques from optic and genetics are used to control for example the activity of neurons (Deisseroth et al. 2006). Many years ago, the study of the brain was quite slow and invasive but from the 1970 the discovery of a family of proteins called opsins was crucial for such research. The opsins are a group of light sensitive proteins which can be found in the mammalian retina as well as in some microorganisms. These latter respond to light by regulating the movement of ions into and out of cells (Oesterhelt and Stoeckenius, 1971; Matsuno-Yagi and Mukohata, 1977; Harz and Hegemann, 1991). This is quite similar to what happen to neurons for transmitting brain signals. Many studies have been conducted for integrating the opsins to the brain of mice. Finally, it has been found that engineered proteins related to the family of the channelrhodopsin are able to turn neurons on or off in very brief time (Zhang et al. 2011; Deisseroth 2011; Packer et al., 2013). The optical control of neurons in model organism by microbial sensors is helping scientist to reveal how the brain works. Nowaday photoactivated

proteins are used for a wide range of applications. For example, in controlling the production of the second messenger cAMP involved in intracellular signal transduction. Light-regulated adenylyl cyclases which are called photoactivated adenylyl cyclases (PAC) come from different small microorganisms and can be used in higher eukaryotes. An example are the two PACs of the unicellular flagellate *Euglena gracilis* (euPACs) and the PAC which derives from the bacterium *Beggiatoe* (bPAC). They are modular and consist of a photoreceptor fused to an enzyme. Different kind of receptor domains do exist, the BLUF domain (blue light receptor using FAD) called F and the LOV domain (light, oxygen, voltage). *Beggiatoa* is a sulfide-oxidizing bacterium that colonizes large areas of sea ground in the form of microbial colonies. Its bPAC is a 350 amino acid protein with a photoreceptive BLUF domain (F) and a catalytic domain (C) (Stierl et al., 2010) (Figure 11 A).

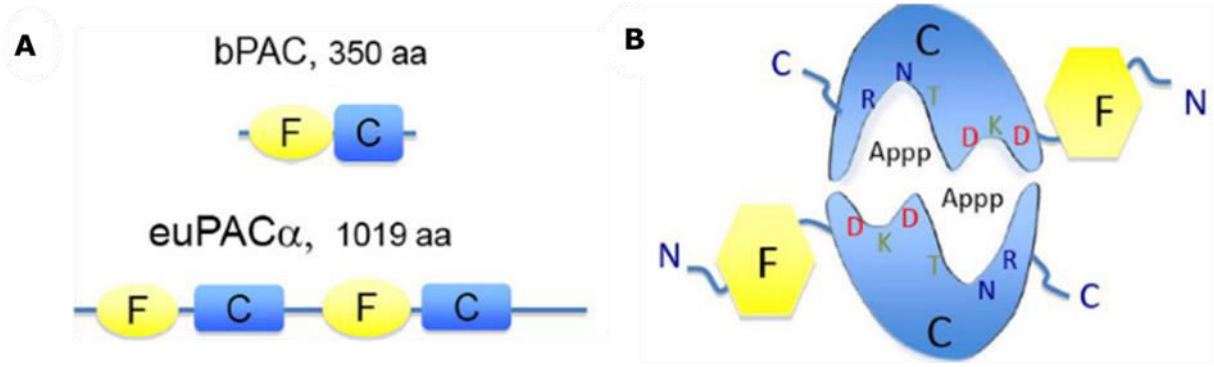

Figure 11: The photo-activated adenylyl cyclase from *beggiatoa* (bPAC)

A) In the upper part the organization of the photoreceptive BLUF domain (*F*) and the catalytic domain (*C*), in the lower part there is the representation of the light regulated adenylyl cyclase euPACα. **B)** Absorption of light by flavin-binding BLUF domain (F) leads to a change in the structure of the catalytic domain (C) (Stierl et al., 2010).

When the photoreceptor BLUF domain adsorbs photons, there is a structural rearrangement which results in activation (Stierl et al., 2010) (Fig 11 B). It has been used in different organisms included *Saccharomyces cerevisiae* (Stierl et al., 2010 Trauth bachelor work, 2016). In particular, the bachelor student tested the effect of photoactivated adenylyl cyclase bPAC for functionality in *Saccharomyces cerevisiae*. More specifically Trauth tested the influence of the optogenetic tool on PKA signal transduction pathway during sporulation. His work demonstrated that activation of bPAC affects cellular growth and entry in meiosis, therefore sporulation is prevented. It was concluded that PKA activation which leads to growth disorder can be regulated by light regulated adenylyl cyclase. To reinforce this assess recently it has been published an article which demonstrates that by expressing this bacterial protein in yeast cells, high-resolution temporal control of PKA activity can be achieved (Stewart-Ornstein et al. 2017).

1.7 The aim of this work

The main goal of the project was to understand how spore formation is regulated by PKA. The adaptation of the spore numbers to nutrients is performed at the level of the MP in meiosis II. PKA has many targets which regulates by phosphorylation. Which of these targets are connected with sporulation and spore number control? A central question was to know if the MP components Mpc54, Mpc70 and Spo74 plus the protein Ady1 are regulated by PKA and if this affects the number of spores. Examples of PKA targets are transcription factors which could bind the promoter of the genes of the MP components, thus another goal was to see if PKA can regulate the expression of the MP components by regulating the transcription factors and if this results in a phenotype. Ady1, a possible sporulation-specific factor under the control of the Ras/cAMP/PKA pathway was another candidate to study to know if PKA affects the spore number control by influencing the activity of such protein. Spore formation is regulated by carbon dioxide, therefore a test to see if this metabolite of the carbon metabolism affects the spore numbers at the level of the MP components was performed. The goal was to find the molecular reasons for the changes in sporulation. PKA has targets which are involved in other signal transduction pathways. In this work, it has been tested if they are relevant for the spore formation as well.

2 Results

2.1: PKA regulates spore numbers

The Ras/cAMP/PKA signaling pathway regulates the entry into meiosis (Donzeau and Bandlow., 1999; Matsuura et al, 1990). The influence of the signal transduction pathway on the meiotic cell division and on the regulation of the number of spores has been recently investigated. In our laboratory, it has been shown that the regulation of spore formation by acetate takes place after commitment to meiosis and depends on PKA and appropriate A kinase activation by Ras/Cyr1 adenylyl cyclase (Jungbluth et al. 2012). The aim is to understand how the spore number is regulated by using mutants in the Ras/cAMP/PKA pathway.

2.1.1: Low PKA activity affects the abundance of two MP components

In the experiments performed in this work the MP components Mpc54, Mpc70 and Spo74 of the yeast strains, were labeled with GFP at the C-terminus. Afterwards, a series of time course analysis of the new created mutants together with control strains were performed. A mutant strain in which Mpc54 was tagged with GFP sporulated as expected, mostly triads and tetrads were found at 0.1 and 1% of KOAc while mostly dyads were found at 0.01% of KOAc (Figure 12 A). An increase of spore formation and sporulation efficiency was found at low concentration of potassium acetate in *cyr1*^{K1712A} mutant (Figure 12 B, C). Afterwards western blotting and quantification of Mpc54-GFP demonstrated no differences in the abundance in the mutant compared to the control (Figure 12 D, E).

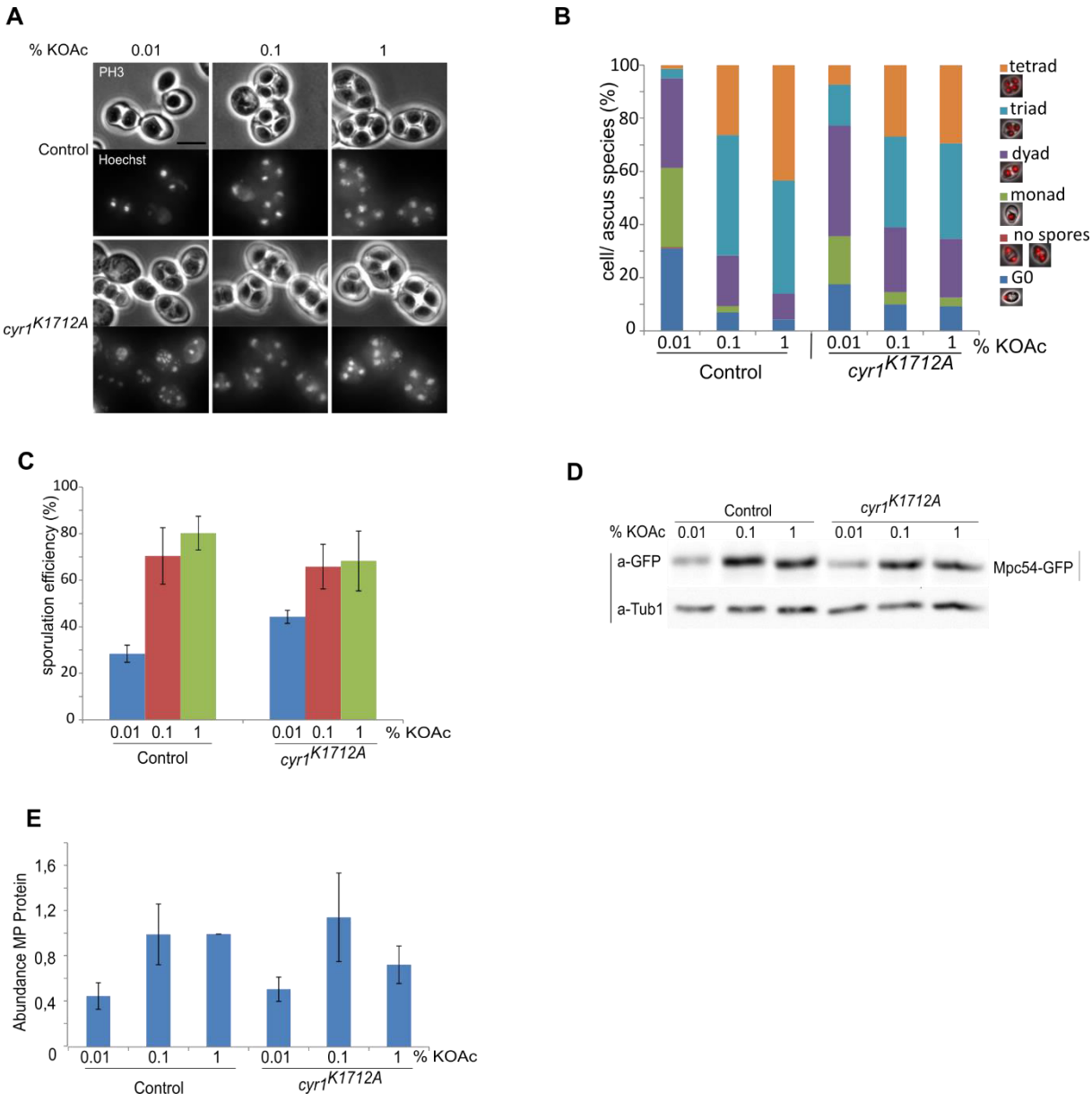

Figure 12: Influence of *cyr1*^{K1712A} mutation on the abundance of Mpc54-GFP during meiosis

A) Light and fluorescence microscope analysis of a control strain *CYR1* (YMM14) and the mutant strain *cyr1*^{K1712A} (YMM23). Sporulated cells in liquid medium were stained with Hoechst 33342 and subjected to microscope analysis. Bright field (PH3) and images taken with DAPI fluorescence filter are shown. Scale bar corresponds to 5 µm. **B)** Quantification of the phenotypes observed from the experiments described in A). Between 100 and 200 cells were assessed for each strain and each condition. Each strain was measured at least three times. In the evaluation were considered the following cell species; cells that do not initiate meiosis (G0), cells that initiate meiosis but did not form spores (no spores) and cells with four (tetrads), three (triads), two (dyads) or one spore (monad). Bar corresponds to 5 µm). **C)** Sporulation efficiency of the strains from the experiment described in B) which directly reflects the number of spores produced by the cells. Each strain was measured at least three times. Standard deviations are indicated by error bars. **D)** Western blot analysis to determine the amount of Mpc54-GFP, in mutant and control strains showed in A). After that sporulation was induced, samples (a corresponding quantity of OD_{600} = 2 of cells) of each strain were collected at one

hour intervals (3 to 8h) and pooled together for SDS-PAGE and immunoblotting. In this way, the total amount of formed proteins can be analyzed. Mpc54-GFP was detected with an anti-GFP antibody. An antibody specific for Tub1 was used to visualize Tub1 as a loading control. **E)** Quantification of the abundance of Mpc54-GFP from the western blot in D) normalized to the loading control Tub1. Each strain was measured at least three times. Standard deviations are indicated by error bars.

Another mutant strain in which Mpc70 was tagged with GFP was tested. Control cells and mutants at 0.1 and 1% of potassium acetate displayed many dyads. At low concentration of nutrients about 30% of control cells sporulated while mutants showed the 70% of sporulated cells (Figure 13 A). Sporulation analysis in this case as well displayed the increased spore formation and sporulation efficiency at 0.01% of KOAc (Figure 13 B, C). It has been also observed that the fusion of GFP to Mpc70 negatively affected the number of spores, mostly dyads and triads were formed (Bajgier et al., 2001). Western blots and quantification analysis of the tested protein showed an increase of the abundance of Mpc70-GFP at low concentration of KOAc in *cyr1*^{K1712A} mutant compared the control (Figure 13 D, E).

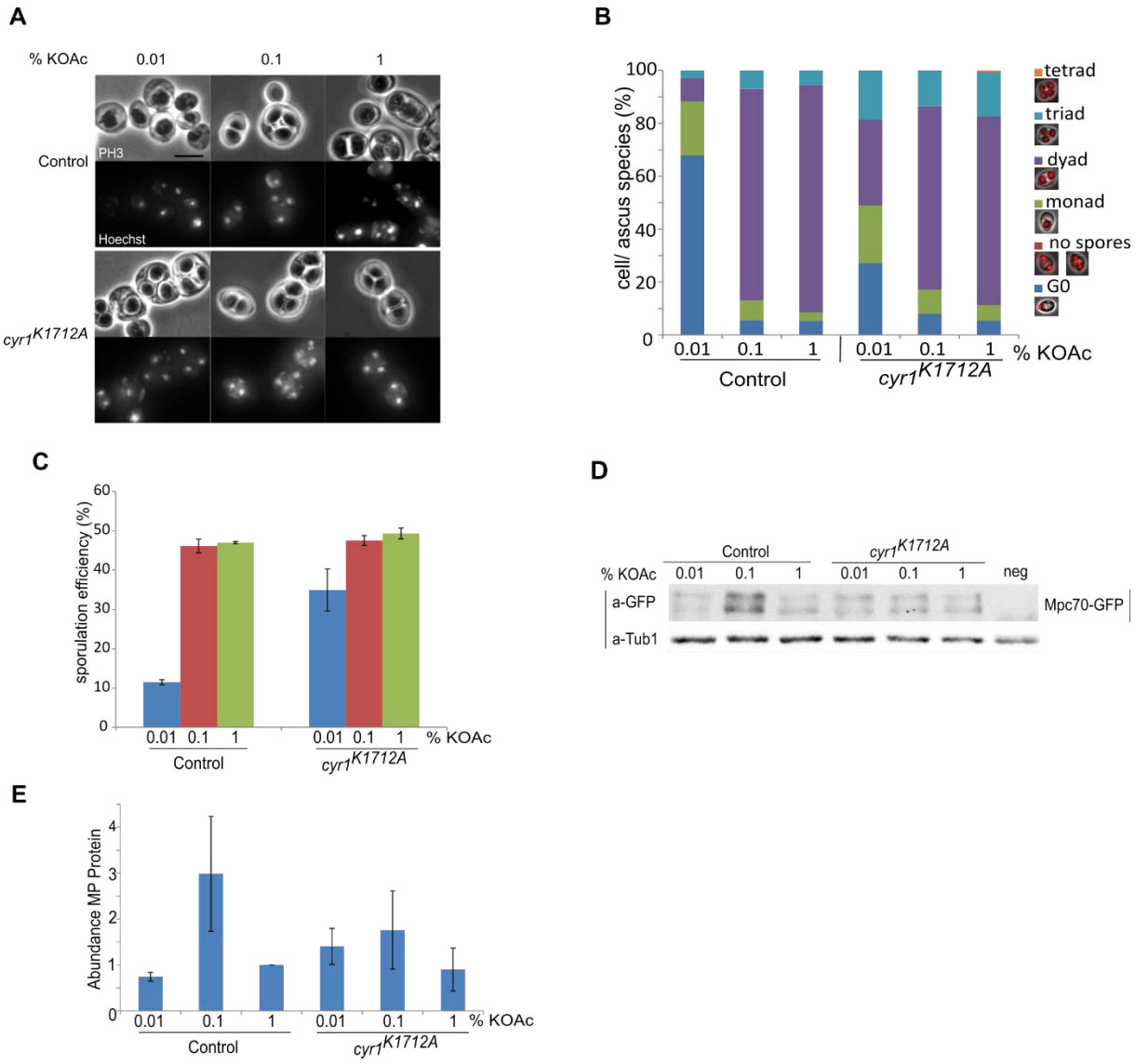

Figure 13: Influence of *cyr1*^{K1712A} mutation on the abundance of a Mpc70-GFP during meiosis

A) Light and fluorescence microscope analysis of a control strain *CYR1* (YMM5) and the mutant strain *cyr1*^{K1712A} (YMM18) performed as described in Fig. 12 A). Bright field (PH3) and images taken with DAPI fluorescence filter are shown. Bar corresponds to 5 µm. **B)** Quantification of the phenotypes observed from the experiments described in A). Implementation is described as shown in the legend of Fig. 12 B. **C)** Sporulation efficiency from the strains described in A). Experimental details as described in the legend of Fig. 12 C. **D)** Western blot analysis to determine the amount of Mpc70-GFP, in mutant and control strains showed in A). As negative control (neg) a wild type strain was used (YKS32). After that sporulation was induced, samples (a corresponding quantity of OD_{600} = 2 of cells) of each strain were collected at one hour intervals (4 to 9h) and pooled together for SDS-PAGE and immunoblotting. In this way, the total amount of formed proteins can be analyzed. Mpc70-GFP was detected with an anti-GFP antibody. An antibody specific for Tub1 was used to visualize Tub1 as loading control. **E)** Quantification of the abundance of Mpc70-GFP from the western blot in C) normalized to the loading control Tub1. Each strain was measured at least three times. Standard deviations are indicated by error bars.

Finally, a strain with Spo74-GFP was assayed. Dyads and triads were found at 0.1 and 1% of nutrients in control and mutant cells. Lot of monads were found at 0.01 % of KOAc in the

control while dyads and triads were found in the mutant (Figure 14 A). Increased spore formation and sporulation efficiency was found at low concentration of KOAc in the mutant (Figure 14 B, C). It has been shown that GFP at the C-terminus of Spo74 reduces its functionality (Bajgier et al., 2001). Regarding the abundance of Spo74-GFP western blot and quantification analysis showed an increase of the protein at low concentration of nutrients in *cyr1*^{K1712A} (Figure 14 D, E).

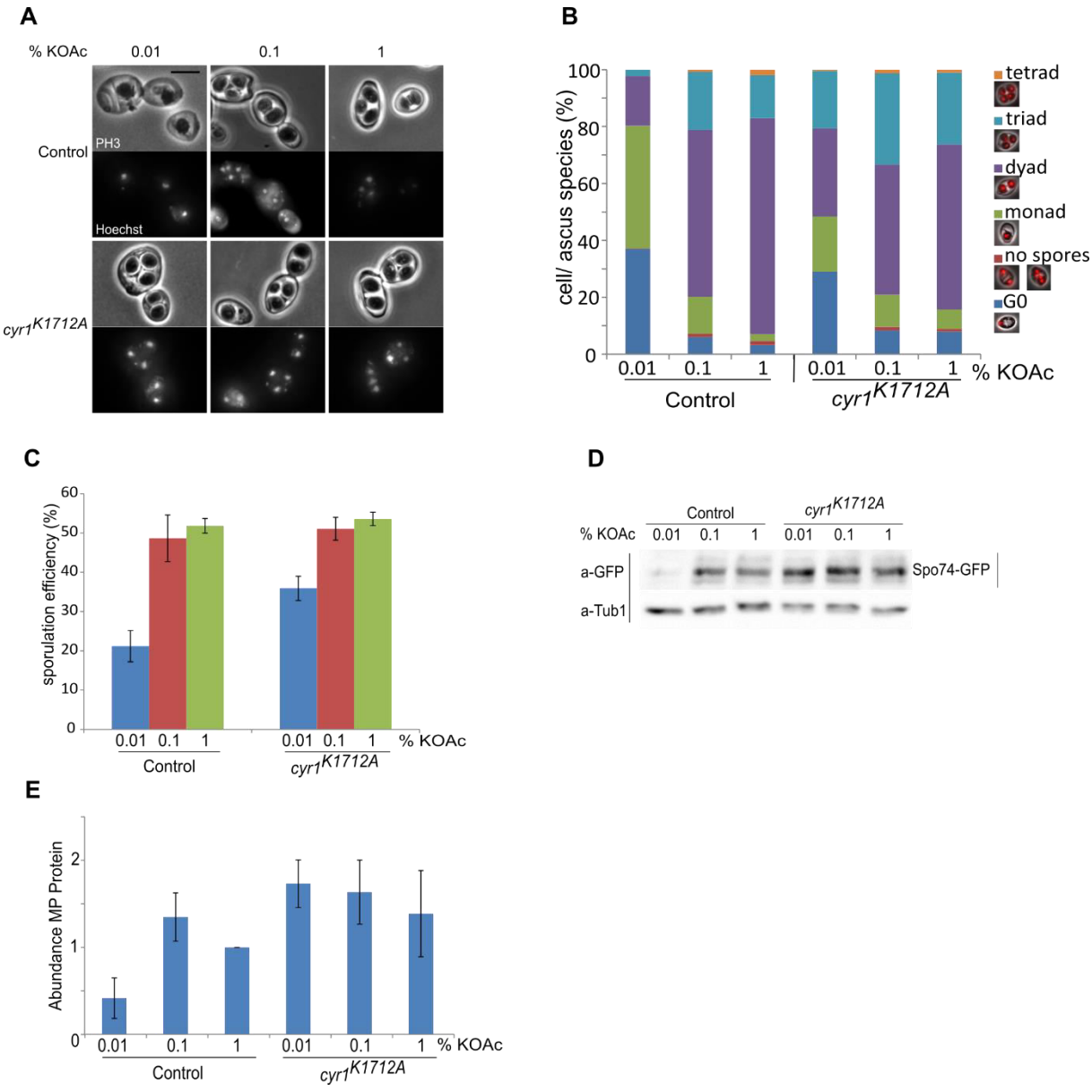

Figure 14: Influence of *cyr1*^{K1712A} mutation on the abundance of Spo74-GFP during meiosis

A) Light and fluorescence microscope analysis of a control strain *CYR1* (YMM6) and the mutant strain *cyr1*^{K1712A} (YMM20) performed as described in Fig. 12 A). Bright field (PH3) and images taken with DAPI fluorescence filter are shown. Bar corresponds to 5µm. **B)** Quantification of the phenotypes observed from the experiments described in A). Experimental details as described in the legend of Fig. 12 B). **C)** Sporulation efficiency from the strains described in A). Implementation is described as shown in the legend of Fig. 12 C). Each strain was measured at least three times. Standard deviations

are indicated by error bars. **D)** Western blot analysis to determine the amount of Spo74-GFP, in mutant and control strains showed in A). Samples for immunoblotting were prepared as observed in Fig. 13 D). An antibody specific for Tub1 was used to visualize Tub1 as loading control **E)** Quantification of the abundance of Spo74-GFP from the western blot in E) normalized to the loading control Tub1. Each strain was measured at least three times. Standard deviations are indicated by error bars.

The experiments showed an increased abundance of the MP components Spo74 and Mpc70 at low concentration of KOAc.

2.1.2: Low PKA activity leads cells to enter earlier in meiosis

MP components are localized to the SPBs. In order to know how PKA affects the localization of the MP components in meiosis, time courses and microscope analysis were performed. Mature MP components tagged to a fluorophore like GFP, can be seen by one to four bright signals. These signals are visible like spots and depend on the number of modified SPBs. Variables like MP formation efficiency, which represents the number of formed MPs, and the number of cells in meiosis, recognizable by the presence of one to four bright GFP spots, were examined. *MPC54* is an early gene. Therefore, to check its localization to the SPB samples have been picked at one hour intervals from the third to the ninth hour in SPO medium. In figure 15 A are represented examples of Mpc54-GFP localized to the SPBs. At 0.01% of KOAc two and three dots are localized to the SPB respectively in the control strain and in the mutant. At 0.1% of KOAc the species represented in both control and mutants were the cells with 3 dots of Mpc54-GFP while at 1% of KOAc the species represented were cells with 4 dots. Such *in vivo* assay revealed that disturbance of the cAMP production by $cyr1^{K1712A}$ mutant leads cells to enter in meiosis II earlier and in higher number at all concentrations of KOAc. In the control, at 0.01% of KOAC, the peak of cells in meiosis appeared after 8 hours in SPO liquid medium while in the mutant the peak of cells in meiosis appeared after 7 hours and in larger number compared to the control. Furthermore, in the mutant at 5 hours there is a similar amount of cells in meiosis as in the control at 8 hours. The MP formation efficiency is quite stable in the control with a slight increase after 8 hours while in the mutant after 7 hours it is already higher than the control (Figure 15 B). In the control at 0.1% of KOAc there is a steady increase of cells in meiosis, while in mutants the peak of cells in meiosis is manifested after 6 hours. The MP formation efficiency is higher and quite stable from the seventh to the ninth hour in the control while in the mutant the higher value is at the sixth hour and decreases during the time course (Figure 15 C). In the cultures with 1% of KOAc the number of cells in meiosis and the MP formation efficiency values are higher

between seven and eight hours while in the mutant the two variabilities have the peaks at the sixth hour (Figure 15 D).

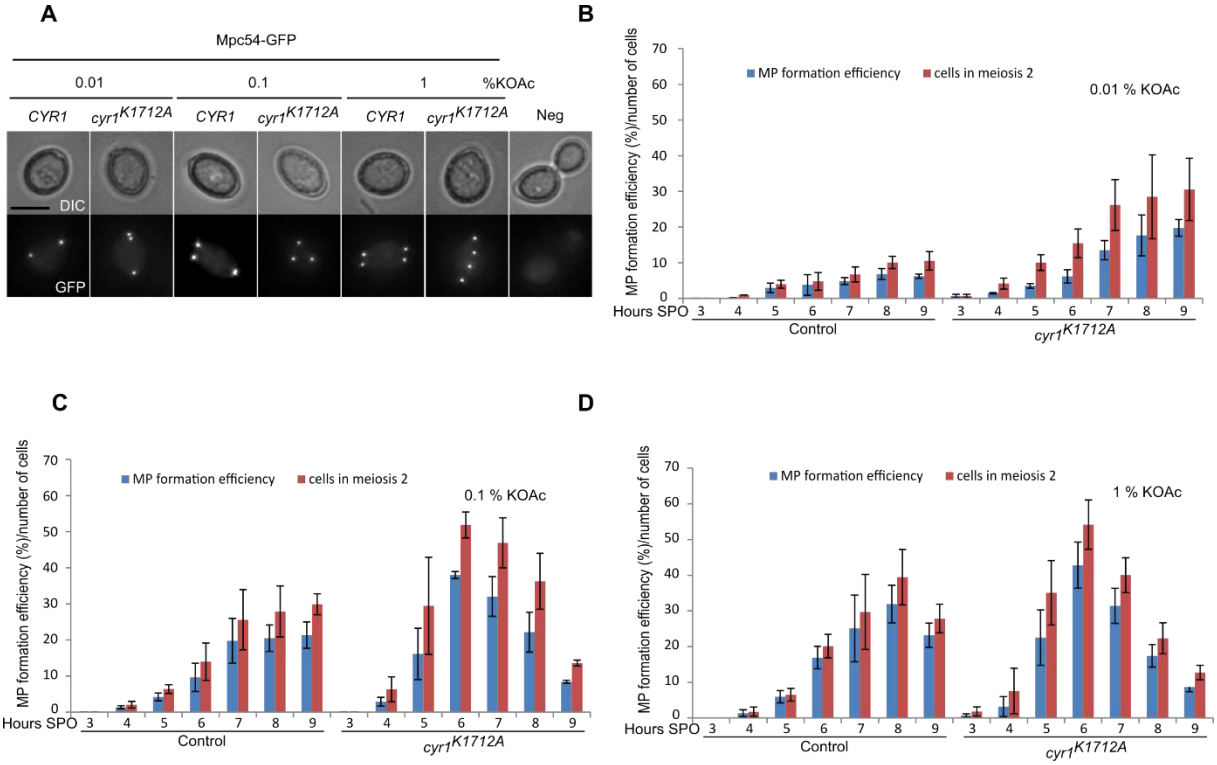

Figure 15: Influence of *cyr1*^{K1712A} mutation on the entry in meiosis in a Mpc54-GFP strain

A) Light and fluorescence microscope analysis of the localization of mature Mpc54-GFP to the SPB was examined in control strain *CYR1* (YMM15) and in the mutant strain *cyr1*^{K1712A} (YMM23) during the course of sporulation. Shown are images with differential interference contrast (DIC) and with GFP filter. Bar corresponds to 5µm. **B)** Localization analysis of the strains in A) was performed with 0.01% KOAc and at one hour intervals (3 to 8h). MP formation efficiency and the exact number of cells in meiosis was calculated at the specific time point. The MP formation efficiency represents the number of formed MPs. Standard deviations are indicated by error bars. **C)** Localization analysis of the strains in A) was performed with 0.1% KOAc and at one hour intervals (3 to 8h). Implementation is described as shown in the legend of Fig. 15 B). **D)** Localization analysis of the strains in A) was performed with 1% KOAc and at one hour intervals (3 to 8h). Experimental details as described in the legend of Fig. 15 B).

The overall result of the figure 15 revealed that low PKA activity affects the entry in meiosis at all concentrations of KOAc in *Saccharomyces cerevisiae* and the number of cells in meiosis at 0.01 % of KOAc. The experiment was performed to check the localization of mature Mpc70-GFP to the SPB. At 0.01% of KOAc one and two spots of Mpc70-GFP are localized to the SPB respectively in the control strain and in the mutant. At 0.1% of KOAc the species represented in both control and mutants were the cells with 2 dots of Mpc54-GFP while at 1% of KOAc the species represented were cells with 3 dots. (Figure 16 A). *MPC70* is a middle gene hence, control and mutant samples were picked at one hour intervals from the fourth to the ninth hour in SPO liquid medium. The test revealed that the GFP signal to the SPB

appears earlier in the *cyr1*^{K1712A} mutant compared to the control. At 0.01% of KOAc there are no peaks of cells in meiosis and MP formation efficiency values either in the control or in the mutant (Figure 16 B). At 0.1% of KOAc there are peaks of cells in meiosis and MP formation efficiency values at five and six hours in the control while in mutant the higher values manifest between four and five hours (Figure 16 C). At 1% of KOAc the highest number of cells in meiosis II and the highest value of MP formation efficiency appear in the control at 7 hours and at 5 hours in the mutant (Figure 16 D).

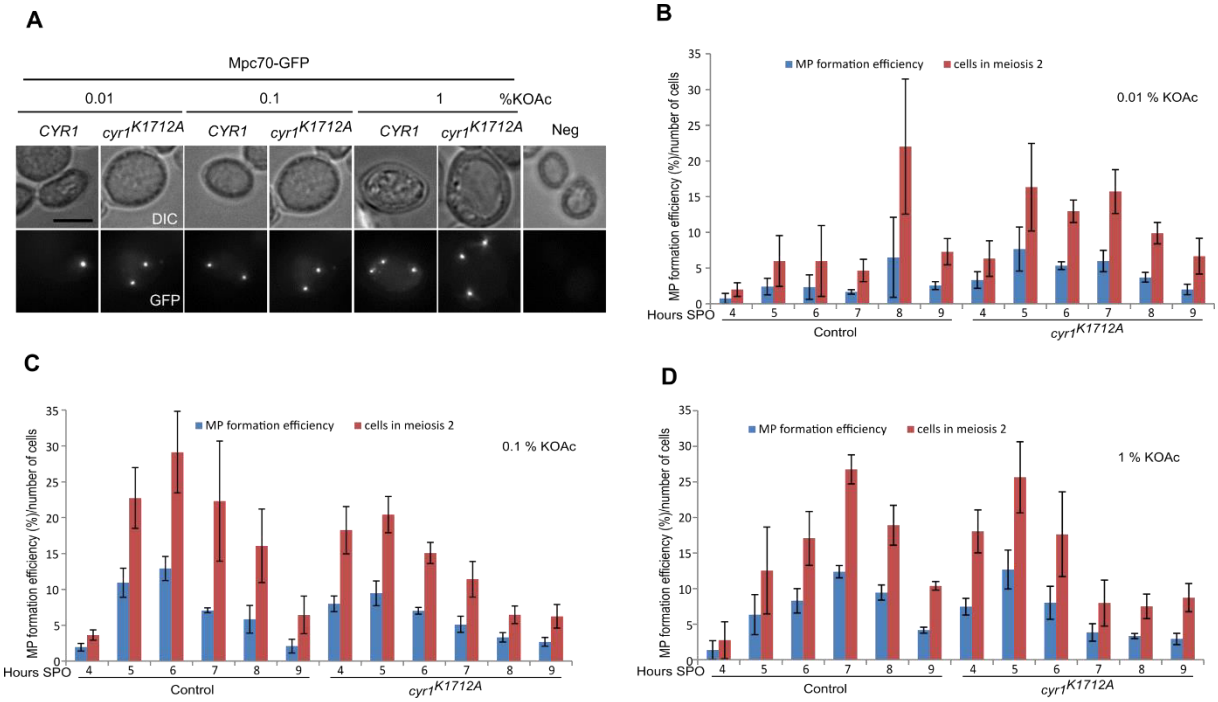

Figure 16: Influence of *cyr1*^{K1712A} mutation on the entry in meiosis in a Mpc70-GFP strain

A) Light and fluorescence microscope analysis of the localization of mature Mpc70-GFP to the SPB was examined in control strain *CYR1* (YMM5) and in the mutant strain *cyr1*^{K1712A} (YMM18) during the course of sporulation. Shown are images with differential interference contrast (DIC) and with GFP filter. Bar corresponds to 5 µm. **B)** Localization analysis of the strains in A) was performed with 0.01% KOAc and at one hour intervals (4 to 9h). MP formation efficiency and the exact number of cells in meiosis was calculated at the specific time point. The MP formation efficiency represents the number of formed MPs. Standard deviations are indicated by error bars. **C)** Localization analysis of the strains in A) was performed with 0.1% KOAc and at one hour intervals (4 to 9h). Experimental details as described in the legend of Fig. 15 B). **D)** Localization analysis of the strains in A) was performed with 1% KOAc and at one hour intervals (4 to 9h). Experimental details as described in the legend of Fig. 15 B).

The experiment showed that low activity of PKA induced cells to enter earlier in meiosis. Furthermore, the number of cells in meiosis is higher in the mutant at 0.01 % of KOAc. Last protein to be controlled was Spo74 (Figure 17 A). As *MPC70*, *SPO74* is a middle gene therefore, samples were picked up at one hour intervals from the fourth to the ninth hour in

SPO liquid medium. At 0.01% of KOAc two and three dots are localized to the SPB respectively in the control strain and in the mutant. At 0.1% of KOAc the species represented in both control and mutants were the cells with 2 dots of Spo74-GFP while at 1% of KOAc the species represented were cells with 3 dots (Figure 17 A). Mutant cells enter in meiosis earlier than control cells. Precisely at 0.01% of KOAC the peak of cells in meiosis II as the highest value of MP formation efficiency appear in the control after 8 hours in SPO liquid medium while they appear in the mutant after 6 hours (Figure 17 B). At 0.1% of KOAc the two values appear after 5 hours in SPO liquid medium in the control while in the mutant they manifest after 4 hours. (Figure 17 C). With 1% of KOAc the number of cells in meiosis II and the MP formation efficiency are higher after the sixth hour in the control while they are higher and quite constant already after the fourth hour (Figure 17 D).

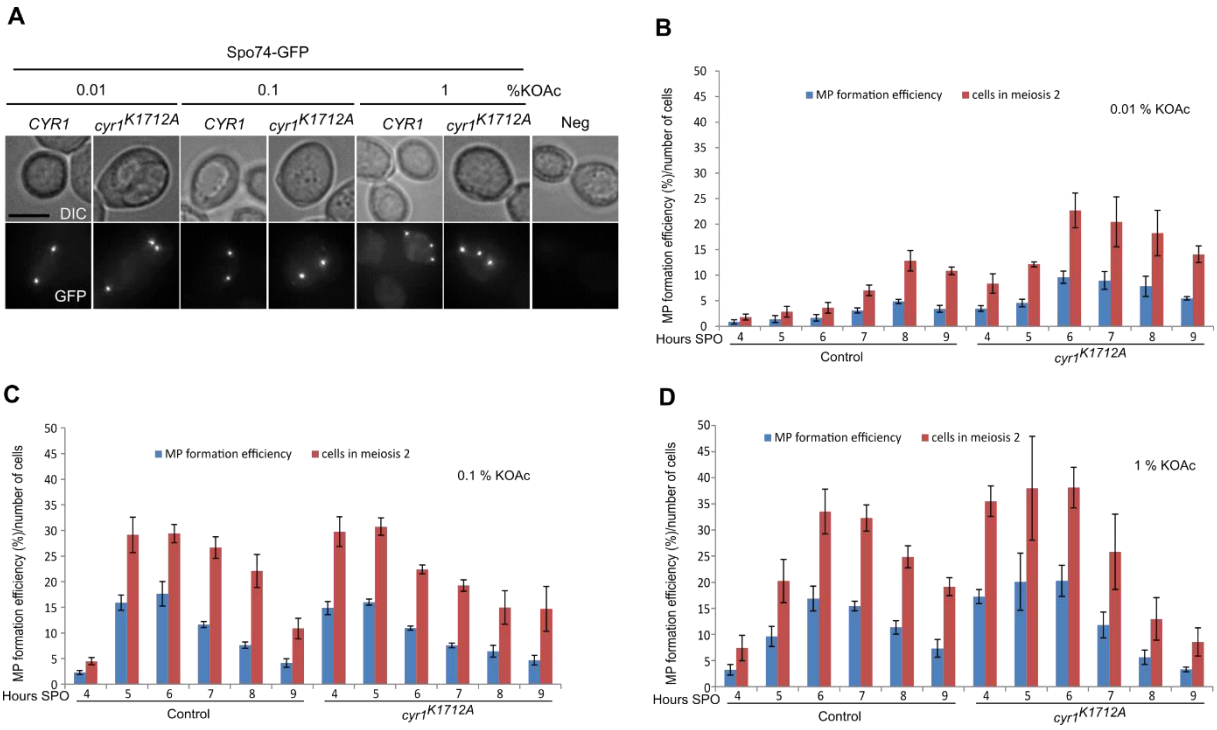

Figure 17: Influence of *cyr1*^{K1712A} mutation on the entry in meiosis in a Spo74-GFP strain

A) Light and fluorescence microscope analysis of the localization of mature Spo74-GFP to the SPB was examined in control strain *CYR1* (YMM6) and in the mutant strain *cyr1*^{K1712A} (YMM20) during the course of sporulation. Shown are images with differential interference contrast (DIC) and with GFP filter. Bar corresponds to 5 µm. **B)** Localization analysis of the strains in A) was performed as described in the legend of the Fig. 16 B). Experimental details as described in the legend of Fig. 15 B). **C)** Localization analysis of the strains in A) was performed as described in the legend of the Fig. 15 B). Experimental details as described in the legend of Fig. 15 B). **D)** Localization analysis of the strains in I) was performed as described in the legend of Fig. 16 B). Experimental details as described in the legend of Fig. 15 B).

Such *in vivo* assay revealed that in *cyr1*^{K1712A} mutants the mature MP proteins appear earlier to the SPB compared to control cells. This phenomenon occurred at all concentrations of nutrients. At low concentration of nutrients is also possible to see that the number of cells which enter meiosis is higher in the mutants compared to the controls. With such experiment, it could be explained that PKA affects the timing and the meiotic entry in *Saccharomyces cerevisiae*.

2.1.3: Reduced PKA activity affects the production of Ady1 in meiosis

The effect of the reduction of the production of cAMP on Ady1 in meiosis was tested. Therefore, yeasts bearing the variant *cyr1*^{K1712A} and control strains have been subjected to time course analysis. Ady1 has been labeled with GFP. The analysis showed that strains sporulated as expected. In control cells at 0.01% of potassium acetate 40% of monads were found while in the mutant the number of monads decreased and the number of dyads and triads increased. Mutants and controls at higher concentrations of nutrients showed dyads and triads at 0.1% of KOAc and triads and tetrads at 1% of KOAc (Figure 18 A). An increase of spore formation and sporulation efficiency was found at low concentration of potassium acetate in the *cyr1*^{K1712A} mutant compared to the control (Figure 18 B, C).

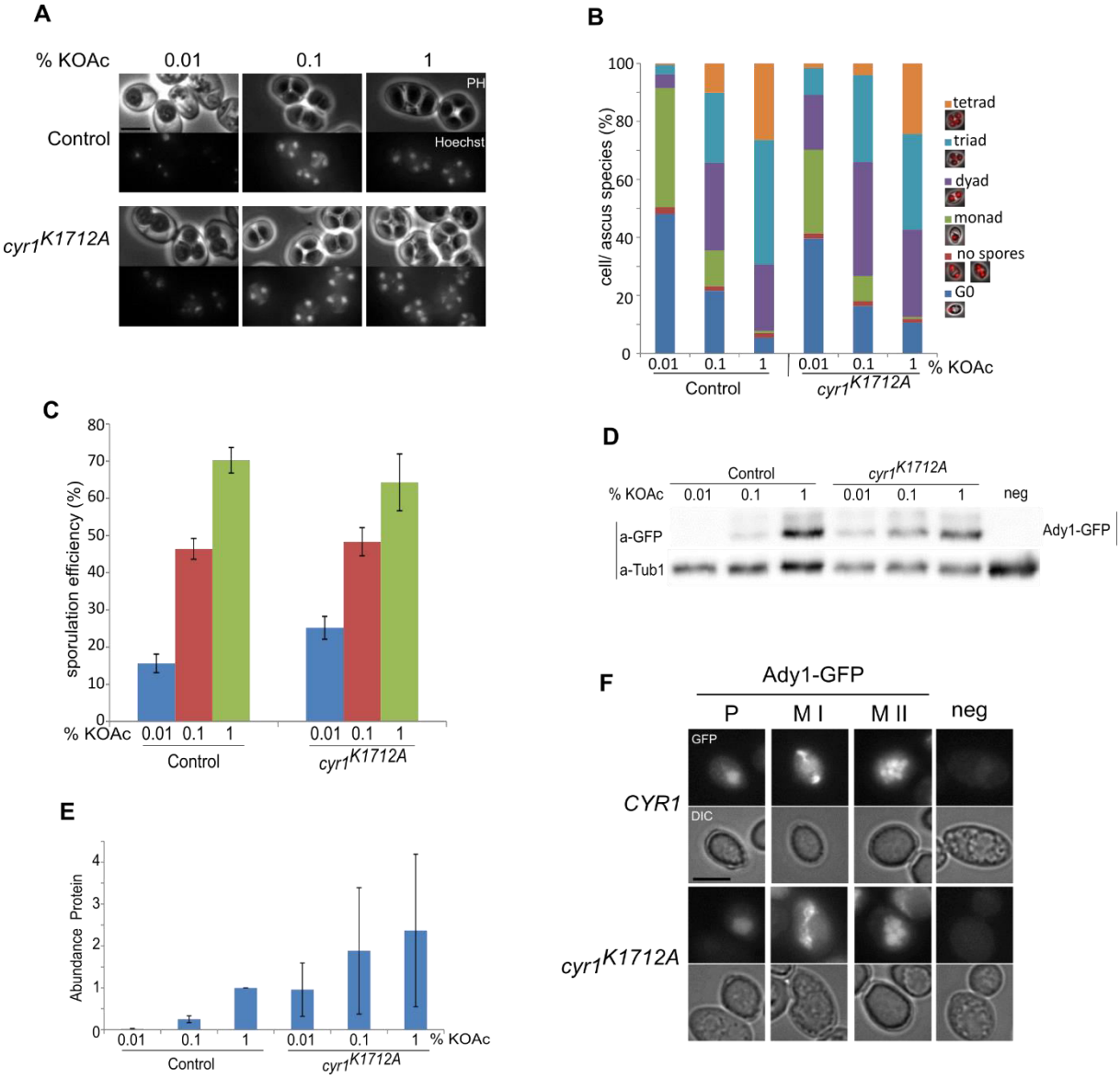

Figure 18: Analysis of the influence of *cyr1*^{K1712A} mutation on a Ady1-GFP strain in meiosis

A) Light and fluorescence microscope analysis of a control strain *CYR1* (YMM15) and the mutant strain *cyr1*^{K1712A} (YMM24) performed as described in Fig. 12 A). Bar corresponds to 5 µm. **B)** Quantification of the phenotypes observed from the experiments described in A) Experimental details as described in the legend of Fig. 12 B). **C)** Sporulation efficiency from the strains described in A). Experimental details as described in the legend of Fig. 12 C) **D)** Western blot analysis to determine the amount of Ady1-GFP, in mutant and control strains showed in A). As negative control (neg) a wild type strain was used (YKS32). Samples for immunoblotting were prepared as observed in Fig. 12 D). An antibody specific for Tub1 was used to visualize Tub1 as a loading control **E)** Quantification of the abundance of Ady1-GFP from the western blot in D) normalized to the loading control Tub1. **F)** The localization of GFP was examined in control (YMM15) and mutant (YMM24) strains, images of living cells were analyzed by fluorescence microscopy to detect GFP signals during meiosis. A negative control is present as well. Images with differential interference contrast (DIC) microscopy are included as a reference. Each strain was measured at least three times. Standard deviations in C) and E) are indicated by error bars.

Next, the amount of Ady1 protein was tested. Western blots and quantification analysis showed that the production of Ady1 is well regulated by the amount of nutrients in the

control. Furthermore, an increase of the production of Ady1-GFP at low concentration of KOAc in the *cyr1*^{K1712A} mutant compared to the control was found. The test displayed that the abundance of Ady1 is affected by PKA (Figure 18 D, E). Deng and Saunders in 2001 found Ady1 localized to the nucleus of mononucleate meiotic cells. Here a localization analysis of Ady1-GFP was performed in control and mutant strains during the time course analysis. In figure 18 F) localization analysis showed that Ady1-GFP signals are typical for a nuclear localization in meiotic cells during the course of sporulation. All of this implies a role of PKA in the control of the amount of Ady1 in meiosis.

2.1.4 Effect of bicarbonate on the spore formation

An experiment to test how the bicarbonate regulates the spore formation was performed. Three strains in which the essential MP components genes *MPC54*, *MPC70* and *SPO74* are labeled with GFP, were subjected to sporulation analysis. In figure 19 A is possible to see a representation of cells sporulated with 0.1% of KOAc with and without HCO_3^- 0.1 mM. An Mpc54-GFP strain showed triads and tetrads, while the Mpc70_GFP and the Spo74-GFP strains showed mostly monads and dyads.

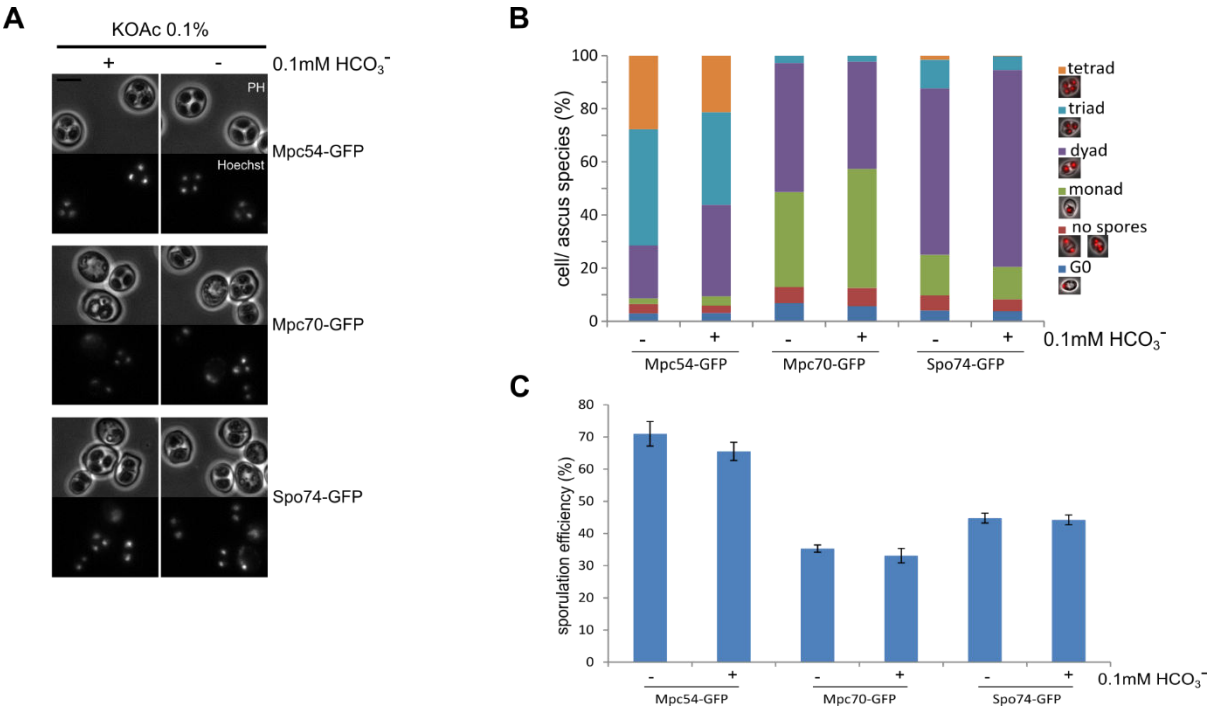

Figure 19: Effect of bicarbonate on sporulation

A) Light and fluorescence microscope analysis of a control strains with respectively Mpc54, Mpc70 and Spo74 labeled with GFP (YCT735, YAM281 and YUK63). Cells sporulated in liquid medium in the presence of 0.1% potassium acetate and in the absence (-) or presence (+) of bicarbonate in

solution. Conditions were as described in the legend of Fig. 12 A). Bar corresponds to 5µm. **B)** Quantification of the phenotypes observed from the experiments described in A). Experimental details as described in the legend of Fig. 12 B). **C)** Sporulation efficiency from the strains described in A). Experimental details as described in the legend of Fig. 12 C). Each strain was measured at least three times. Standard deviations are indicated by error bars.

The analysis did not show any difference in the spore formation and sporulation efficiency between cells sporulated in presence of bicarbonate and cells sporulated in absence of bicarbonate (Figure 19 B, C). Western blot analysis and quantification of all the MP proteins did not show any difference in the abundance of MP proteins compared to the control. In figure 20 A and B is possible to see the western blotting and the quantification of Mpc54 of the two cultures sporulated in presence and in absence of bicarbonate, at each time point, from the sixth to the ninth hour. For the MP component Mpc70 is shown the western and the protein quantification of the samples picked after seven hours until the ninth hour in KOAc, in presence and in absence of bicarbonate (Figure 20 C, D). As for Mpc70, Spo74 was tested after 7 hours in KOAc and western blotting and quantification analysis of the protein did not show any difference in presence or in absence of bicarbonate (Figure 20 E, F).

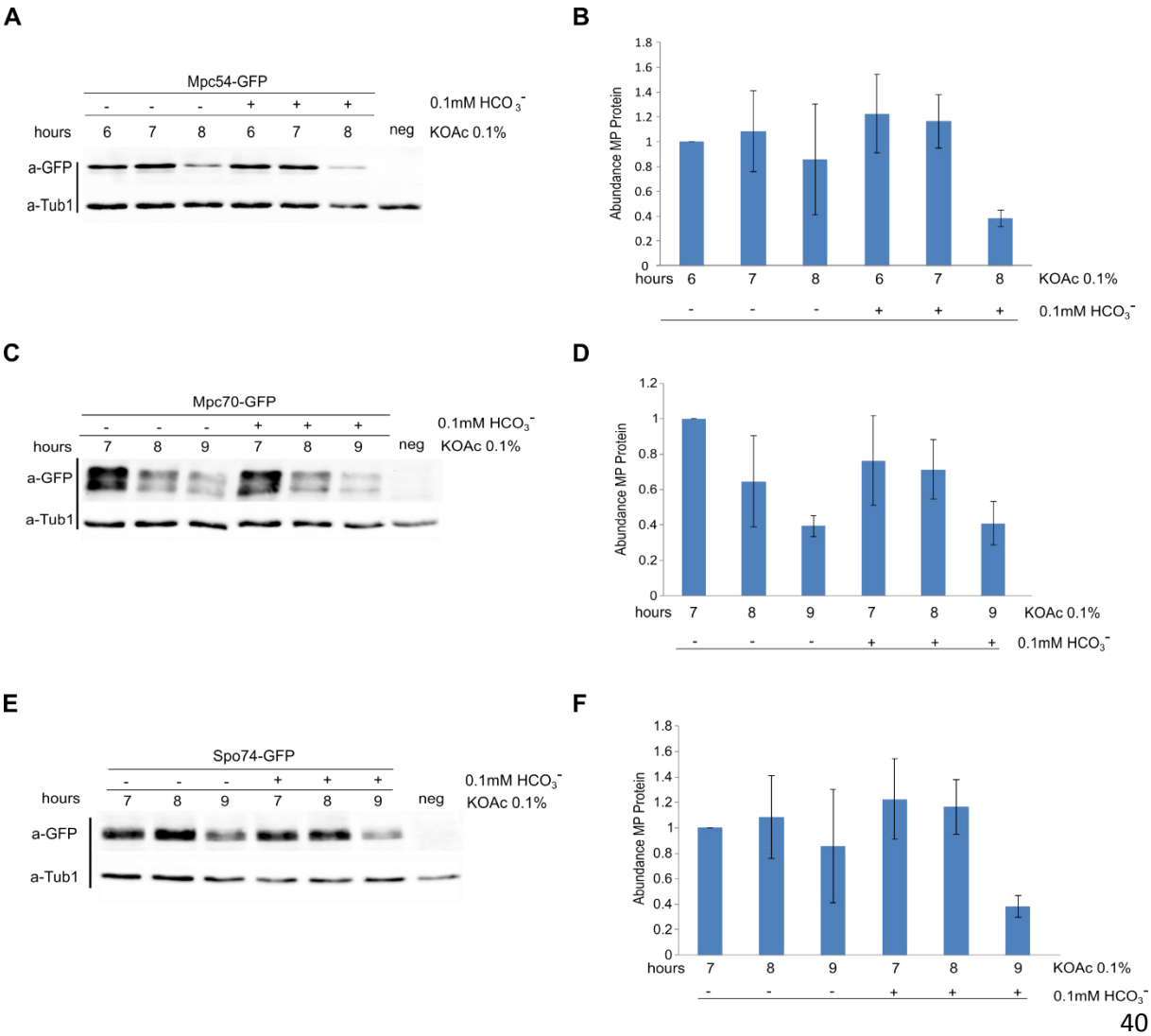

Figure 20: Effect of bicarbonate on MP components during spore formation

A) Western blot analysis to determine the amount of Mpc54-GFP in the strain YCT735, in presence and in absence of bicarbonate. As negative control (neg) a wild type strain was used (YKS32). After 5 hours in SPO liquid medium a corresponding quantity of OD600 2 of cells was collected and subjected to immunoprecipitation. The main culture was shifted in two flasks in which in one of them was added bicarbonate in solution 0.1mM and sailed. Afterwards cells of each flask were collected at one hour intervals (6 to 8h) for SDS-PAGE and immunoblotting. **B)** Quantification of the western blotting in A) Mpc54-GFP was detected with an anti-GFP antibody. An antibody specific for Tub1 was used to visualize Tub1 as a loading control. Quantification of the abundance of Mpc54-GFP was normalized to the loading control Tub1. **C)** Western blot analysis to determine the amount of Mpc70-GFP in the strain YAM281, in presence and in absence of bicarbonate. Sample were collected between 7 and 9 hours and prepared as observed in Fig. 20 A) **D)** Quantification of the western blotting in C) Mpc70-GFP was detected with an anti-GFP antibody. An antibody specific for Tub1 was used to visualize Tub1 as a loading control. Quantification of the abundance of Mpc70-GFP was normalized to the loading control Tub1. **E)** Western blot analysis to determine the amount of Spo74-GFP in the strain YUK63, in presence and in absence of bicarbonate. Sample were prepared as observed in Fig. 20 B) **F)** Quantification of the western blotting in E) Spo74-GFP was detected with an anti-GFP antibody. An antibody specific for Tub1 was used to visualize Tub1 as a loading control. Quantification of the abundance of Mpc70-GFP was normalized to the loading control Tub1. Each strain was measured at least three times. Standard deviations are indicated by error bars.

The analysis showed no differences in the sporulation behaviour between cells sporulated in presence and in absence of KOAc. Likewise, I have not found differences in the MP components abundance compared to the control. The result of the sporulation here is different compared to the experiment reported by Jungbluth in 2012 to check the effect of the intracellular carbon dioxide/bicarbonate on the spore formation.

2.1.5 Plasma membrane localization of Ras is affected by cAMP levels

In this work the effect of the light activated adenylyl cyclase on the activity of Ras proteins in vegetative and sporulating cells was tested. To do that an *in vivo* reporter for Ras activity was used. It is formed by a Ras-binding domain (RBD) of human Raf1 fused to GFP. Localization analysis was performed in vegetative cells. The typical nuclear and peripheral RBD-GFP signal was found in control cells and in cells which carry the bPAC (Figure 21 A). In the latter, quantification of cells and species showed a slightly higher number of cells with just nucleus localization. After three hours under blue light most of the cells with the bPAC lost their plasma membrane localization (Figure 21 B).

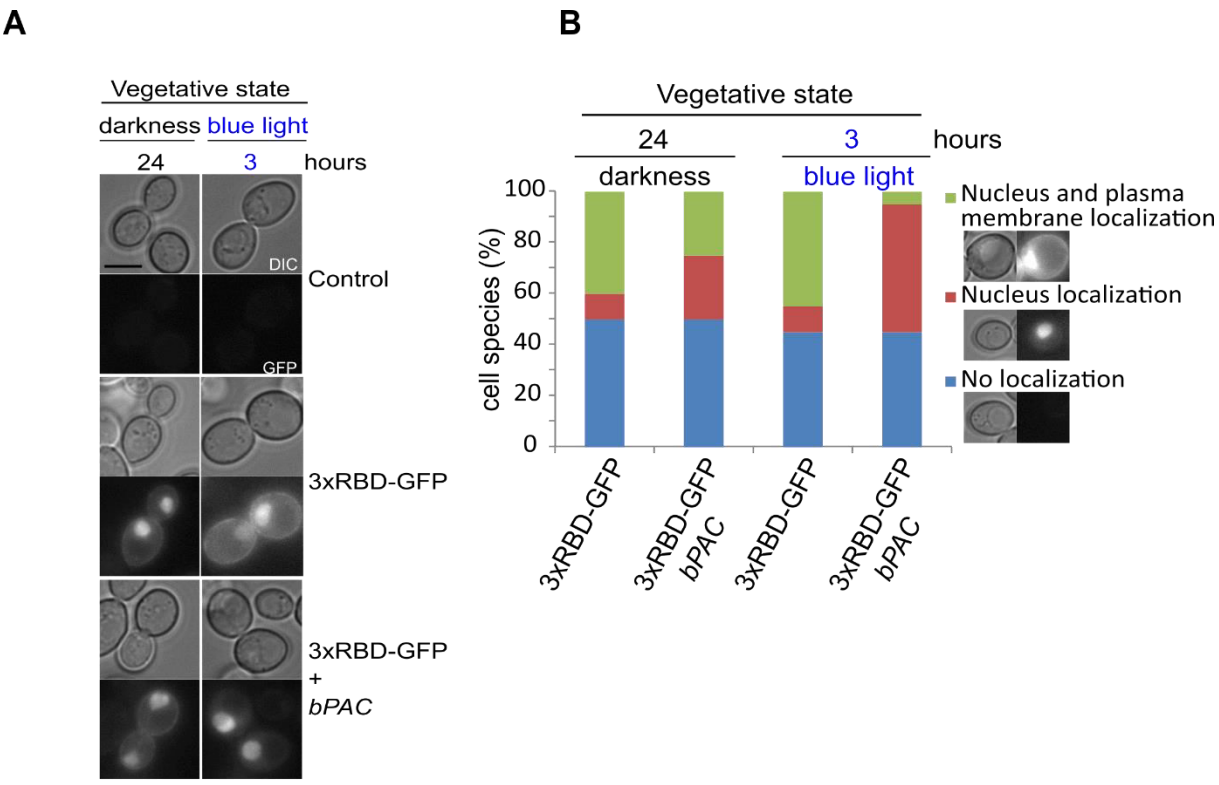

Figure 21: Photoactivated adenylyl cyclase effects plasma membrane localization of Ras in vegetative cells.

A) The localization of a 3xRBD was examined in YCR77 plus an empty plasmid and 3xRBD-GFP and in YCR77 plus 3xRBD-GFP and bPAC during vegetative growth in darkness as well as 3 hours after cells were kept under blue light. DIC and GFP images are shown. Cells carrying two empty plasmids were used as negative control. Bar corresponds to 5µm. **B)** Quantification of cells and species observed in the same experiment

To know the effect of the bPAC on sporulating cells an aliquot of the same vegetative yeast cells in darkness were shifted into SPO liquid medium. Microscope pictures were taken after two hours in SPO medium in darkness, one hour under blue light and twentytwo hours under blue light as well (Figure 22 A). The quantification of cells and species showed that after 2 hours in SPO medium and in darkness, cells had the typical plasma membrane and nucleus localization of RBD-GFP. Moreover, like in the experiment with yeasts in vegetative state, the number of cells with just nucleus localization was higher in the yeasts which carried the bPAC plasmid compared to the control. In addition to this, the analysis demonstrated that after 1 further hour under blue light the number of cells with just nucleus localization increased (Figure 22 B).

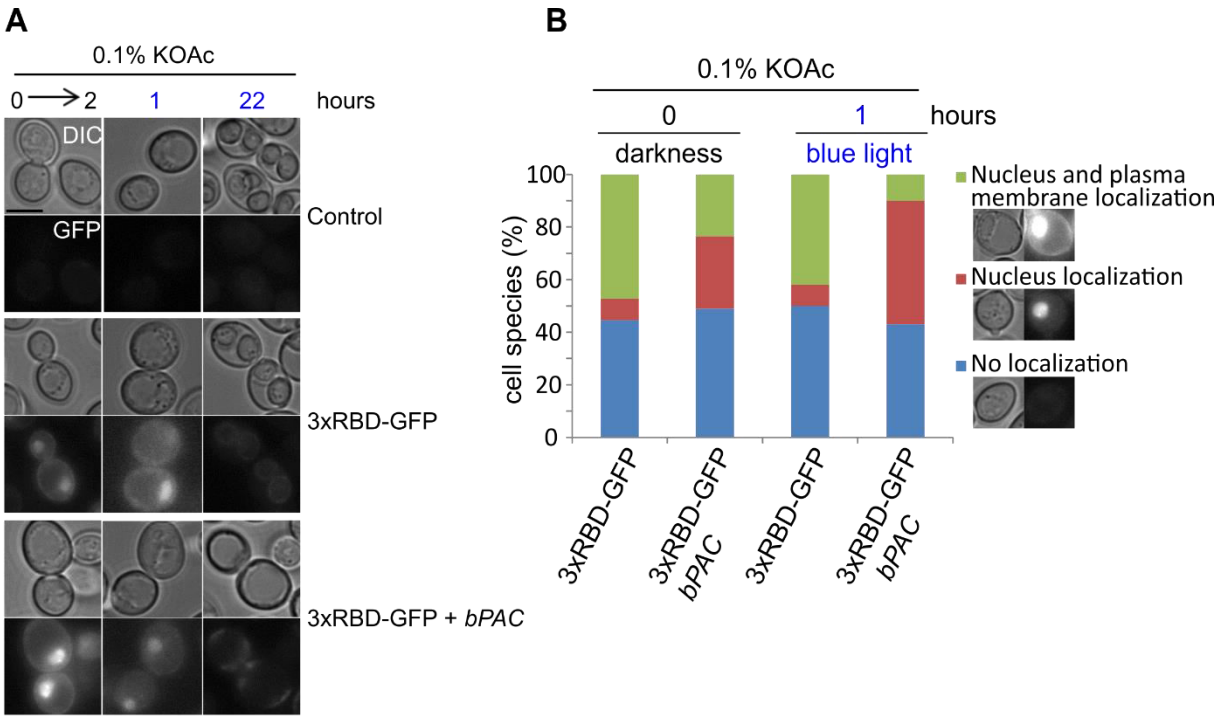

Figure 22: Photoactivated adenylyl cyclase effects plasma membrane localization of Ras in sporulating cells.

A) The localization of a 3xRBD was examined in YCR77 plus an empty plasmid and 3xRBD-GFP and in YCR77 plus 3xRBD-GFP and bPAC in SPO liquid medium after two hours in darkness as well as after one hour that cells were kept under blue light. DIC and GFP images are shown. Cells carrying two empty plasmids were used as negative control. Bar corresponds to 5 µm. **B)** Quantification of cells and species observed in the same experiment.

The experiments showed that cells which carry the photoactivated adenylyl cyclase bPAC when subjected to blue light lose partially their plasma membrane localization of active Ras in the vegetative and in the sporulating state.

2.1.6: Tools to study the effect of the high PKA activity on the regulation of spore formation

The previous experiments which have involved the usage of the *cyr1*^{K1712A} mutation showed that a decrease in the activity of the Ras/cAMP/PKA transduction pathway resulted in the increase of the number of spores at low concentration of nutrients. More precisely the decrement of the production of cAMP led to the increase of two MP components, Mpc70 and Spo74. Therefore, I wanted to address the effect of the high PKA activity on the MP components. In our laboratory, the former PhD student Jungbluth performed an assay to see what is the effect of high PKA on spore formation by hyperactivation of Ras after commitment to meiosis. To do that he used the dominant active *RAS2*^{G19V} variant which

activates the cAMP/PKA pathway (Kataoka et al., 1984; Píchová et al., 1997; Toda et al., 1985). Ras2 is hyperactivated in this mutant because there is a missense mutation, which does not allow the hydrolysis of GTP bound to Ras2. As consequence, the PKA pathway is always activated (Marshall et al., 1997). The $RAS2^{G19V}$ allele was under the control of a *SPS1* promoter, which is active after commitment to meiosis. He has found out that constitutively active Ras2 after entry into meiosis leads to a reduction of spore formation. In the actual work, several experiments where the Ras2 variant was used to study the abundance of the MP components revealed a variable outcome. The reduction of the spore number was not reproducible in all tests. So, it has been decided to check the effect of the high PKA on the sporulation by another way. During the same work Jungbluth used the method that allows meiosis-specific destabilization of proteins called TIPI system. In this way, he could selectively disable components of the signaling pathway during meiosis like the subunit Bcy1 which has an important regulatory role in the activation of the pathway. Bcy1 exercises a negative influence on PKA (Toda et al., 1987). Jungbluth could show that depletion of the regulatory subunit of PKA reduces spore formation. For that reason, the depletion of Bcy1 by TEV degron under three different promoters has been tested in this work as well. Sporulation was induced at 1% concentration of potassium acetate (Figure 23 A). Yeast cells which degrade Bcy1 by using the TEV degron that is under the *SCC1* promoter showed just one single DNA dot when subjected to microscope analysis. Could be that cells did not enter meiosis most probably because the *SCC1* promoter is active only during vegetative growth. Moreover, the two strains with the TEV degron under the two promoters *CYC1* and *ADH1* entered meiosis but just 5% of the cells sporulated, indeed microscopy analysis showed a very high number of cells in meiosis which did not form spores (Figure 23 B). Unfortunately for technical problems the tagging of the MP components and Ady1 with a fluorescent protein was not successful and the study of the abundance and localization of the proteins was not possible. Nevertheless, this experiment reinforced the previous knowledge about the fact that an increase of the PKA activity after commitment to meiosis has a negative effect on the spore formation.

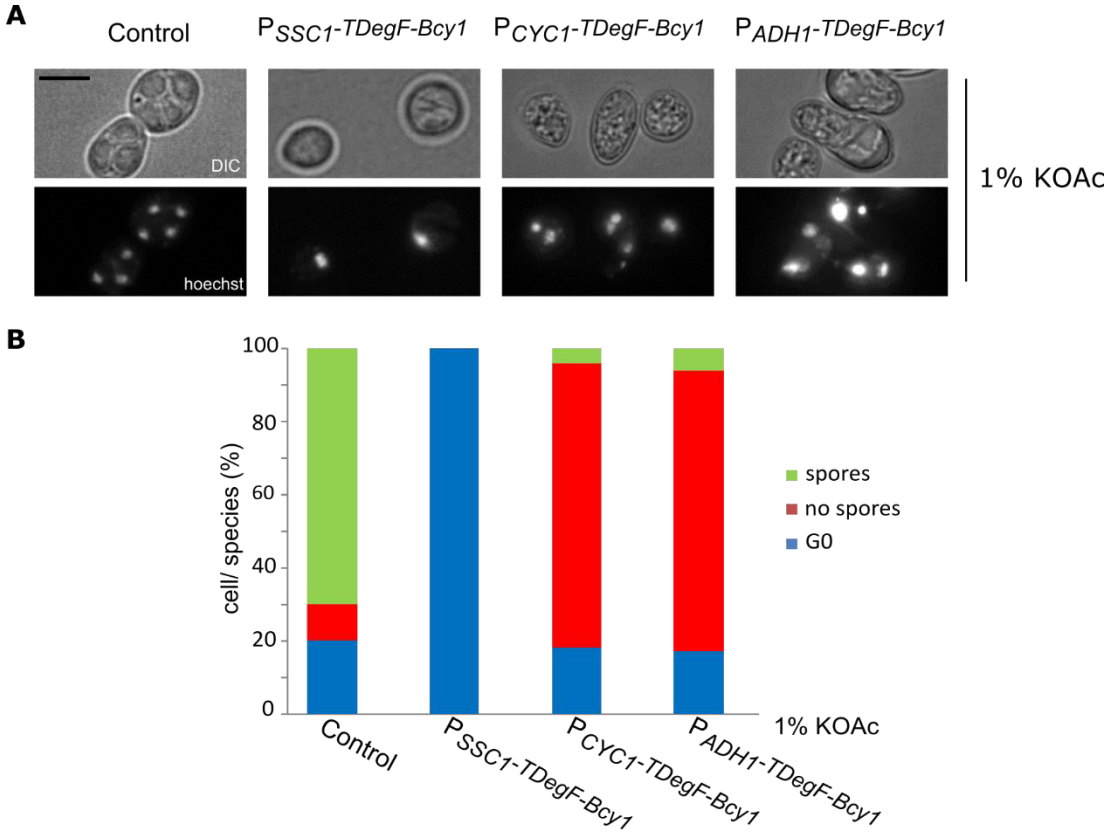

Figure 23: Strong activation of PKA by Bcy1 depletion blocks spore formation

A) Light and fluorescence microscope analysis of Bcy1 depleted cells in meiosis using three different degrons under *SCC1* promoter (YMM34), *CYC1* promoter (YMM64) and *ADH1* promoter (YMM46) plus control cells (YAA146). Sporulated cells in liquid medium were stained with Hoechst 33342 and subjected to microscope analysis. Bright-field (DIC) and fluorescence (Hoechst) images (maximum-intensity projections) are shown. Bar corresponds to 5µm. **B)** Quantification of the phenotypes observed from the experiments described in A). Between 100 and 200 cells were assessed for each strain and each condition. Each strain was measured at least three times. In the evaluation were considered the cells with one single DNA stain (G0), cells that initiate meiosis but did not form spores (no spores) and the cells which formed one to four spores (spores).

Finally, the effect of the photoactivated adenylyl cyclase in course of meiosis was assayed. Control cells and cells with the bPAC were exposed to a blue light intensity of 10 µmol*m^{-2}*s^{-1} (Figure 24 A). In another test cells were exposed to a light intensity of 30 µmol*m^{-2}*s^{-1} (Figure 24 B). Furthermore, I executed a test to see what could happen when yeasts are kept under blue light (30 µmol*m^{-2}*s^{-1}) for a period which goes from the third hour to the seventh hour in sporulation liquid medium. Afterwards cells were shifted in darkness for the remaining 41 hours (Figure 24 C). Sporulation analysis showed that in the three experiments control cells sporulated as expected, mainly tetrads and triads at 1% KOAc, triads and dyads at 0.1% KOAc and dyads and monads at 0.01% KOAc were formed. In the strains with expressed bPAC in all three cases really few cells formed spores or showed

distinguished spots of DNA typical of cells in meiosis. The major part of the cells displayed a single dot that was difficult to classify as cell in G0 phase or prophase.

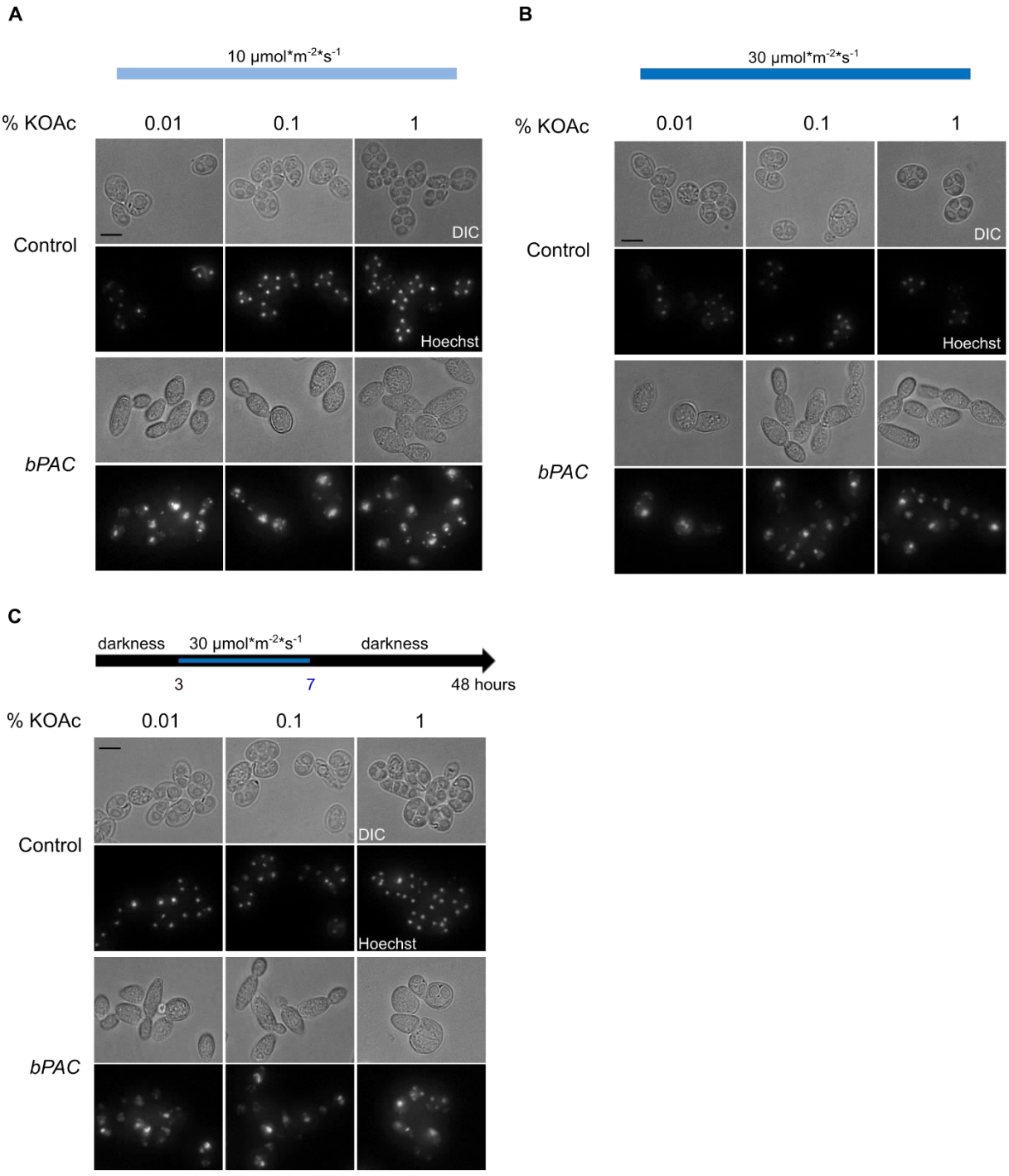

Figure 24: Active PKA achieved by blue light activated bPAC affects spore formation.

A) Light and fluorescence microscope analysis of light regulated adenylyl cyclase (YCR561 + pJT4) and control cells (YCR561 + pRS315). Cells sporulated in darkness for three hours, afterwards were subjected to a blue light intensity 10 µmol*m^{-2}* s^{-1}. Conditions were as described in the legend of Fig. 23 A). **B)** Light and fluorescence microscope analysis of the strains in A). Cells sporulated in darkness for three hours, afterwards were subjected to a blue light intensity 30 µmol*m^{-2}* s^{-1}. Conditions were as described in the legend of Fig. 23 A). **C)** Light and fluorescence microscope analysis of the strains

in A) subjected to a blue light intensity of 30 µmol*m^{-2}* s^{-1} from 3rd hour to 7th hour in SPO liquid medium. Conditions were as described in the legend of Fig. 23 A). Bar corresponds to 5 µm.

More exactly the cells subjected to a blue light intensity of 30 µmol*m^{-2}*s^{-1} did not show any spore after 48 hours in SPO medium. The test demonstrated that bPAC affects the meiotic divisions at all the different conditions of blue light and in a more exacerbated way when the intensity corresponds to 30 µmol*m^{-2}*s^{-1}. Could be that with this approach PKA is induced too early.

2.1.7 High PKA activity induced by bPAC affects the Mpc54 levels in meiosis

Although the experiment in which bPAC was used showed that many cells did not enter meiosis I, a time course analysis was performed to know the amount of the MP component Mpc54 which is encoded by the early gene *MPC54*. A Mpc54-HA yeast strain with the bPAC sporulated under a blue light intensity of µmol*m^{-2}*s^{-1} to see if the complete stop of the spore formation due to the higher intensity has an effect on the protein level. Microscope images confirmed the typical arrest at the initial phases of meiosis in the strain with the bPAC compared to the control while the control sporulated fine with dyads at 0.01% of KOAc, triads at 0.1% and tetrads at 1% of KOAc (Figure 25 A). The examination of the total formed amount of proteins in course of sporulation by western blotting showed a decrease of Mpc54-HA in the strain affected by the bPAC compared to the control (figure 25 B). To know when exactly Mpc54-HA is affected by bPAC during the time course analysis each sample of the different time point were loaded on a SDS gel. After two hours in SPO liquid medium 1% the signal of Mpc54-HA is already present either in control cells or in the cells with bPAC. When cells were subjected to blue light after three hours in KOAc the abundance of Mpc54 decreased in the strain with the photo activated adenylyl cyclase (Fig. 25 C). The test demonstrated that Mpc54 is already formed after the initial phases of meiosis therefore it provides a good model to study the effect of the bPAC. Another experiment with the same Mpc54-HA strains was performed. The main culture with the strain which carried the bPAC was divided in three different cultures and subjected to blue light at three different time points; after one hour, after two hours and again after three hours in KOAc and in darkness. Protein analysis demonstrated that in all cases the decrease of Mpc54 occurred and it was very evident when the tool was activated by blue light after three hours (Fig. 25 D).

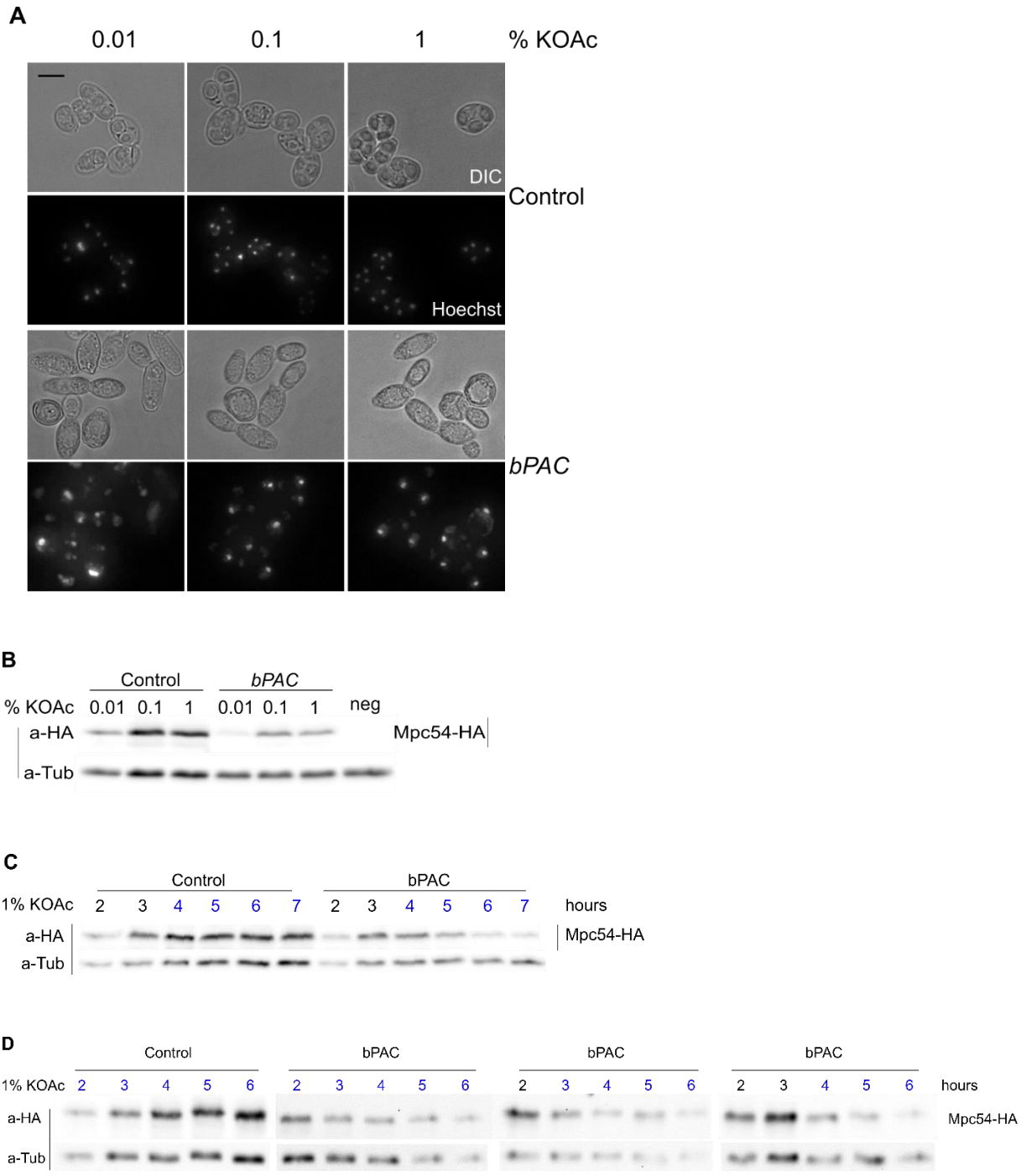

Figure 25: Active PKA achieved by blue light activated bPAC affects Mpc54-HA in meiosis.

A) Light and fluorescence microscope analysis of light regulated adenylyl cyclase (YCR484 + pJT4) and control cells (YCR484 + pRS315). Conditions were as described in the legend of Fig. 24 B). **B)** Western blot analysis to determine the amount of Mpc54 in the strains used for the experiment in A) Mpc54 was tagged with HA. As negative control (neg) a wild type strain was used (YKS32). For SDS-PAGE and immunoblotting, a corresponding quantity of OD_{600} = 2 of cells from each strain was collected at one hour intervals (from the 2^{nd} to 7^{th} hour) and pooled together. In this way, the total amount of formed proteins can be analyzed. Mpc54-HA was detected with an anti-HA antibody. An antibody specific for Tub1 was used to visualize Tub1 as loading control. **C)** Immunoblot analysis of cells used for the experiment in A) during sporulation at 1% KOAc. Cells were taken at the indicated time points after transfer into sporulation medium and subjected to immunoblotting. **D)** Immunoblot

analysis of cells used for the experiment in A) during sporulation at 1% KOAc. Control cells were subjected to blue light after one hour in darkness. Light regulated adenylyl cyclase cells were subjected to blue light at different time points and respectively after one, two and three hours in darkness.

The experiment showed nicely that PKA has an impact on Mpc54 levels in cells which are already in meiosis.

2.1.8 High PKA activity induced by bPAC affects the formation of the proteins encoded by the middle genes *MPC70*, *SPO74* and *ADY1*

A strain with Mpc70-HA in presence of the bPAC and subjected to sporulation under blue light showed the arrest of the cells at the initial phases at all concentrations of KOAc. In figure 26 A is also possible to see the control strain which sporulated fine at all concentrations of nutrients. Western analysis revealed that the abundance of the total amount of Mpc70-HA was not detectable compared to the control (figure 26 B). As it has been done for Mpc54-HA, to understand what is the effect of the bPAC on the protein abundance of Mpc70-HA step by step in meiosis the same samples picked at different time points and used to check the total amount of Mpc70 were singularly loaded on a SDS gel. The signal of Mpc70-HA in the western appeared after the 5th hour in SPO liquid medium 1% while in the strains with expressed bPAC the signal is nearly absent (Fig. 26 C).

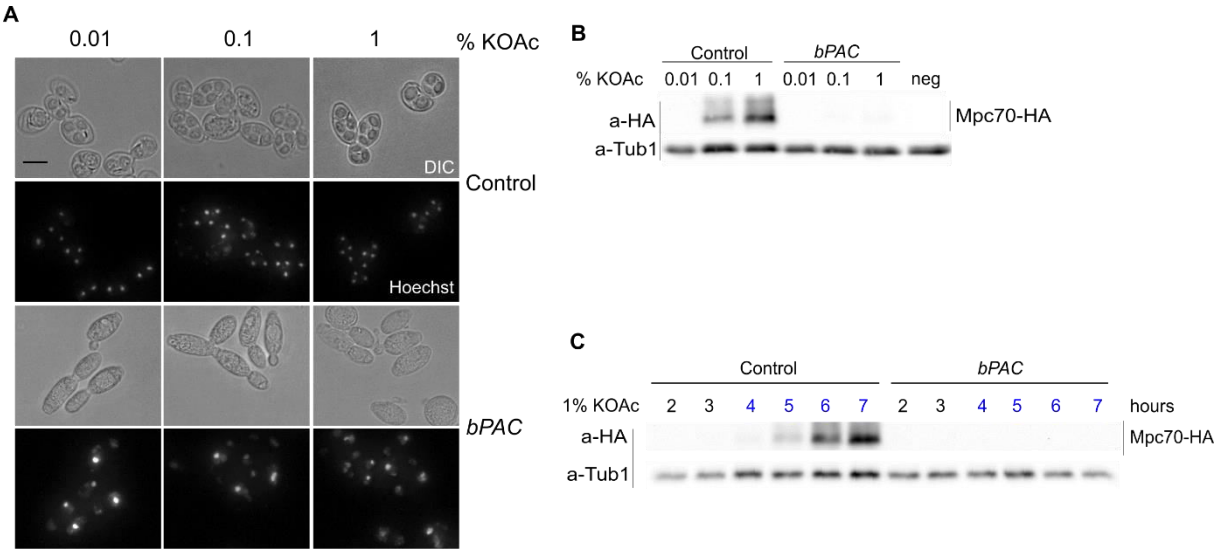

Figure 26: Effect of the high PKA activity induced by bPAC on the abundance of the Mpc70-HA

A) Light and fluorescence microscope analysis of light regulated adenylyl cyclase (YCR485 + pJT4) and control cells (YCR485 + pRS315). Conditions were as described in the legend of Fig. 24 B). **B)**

Western blot analysis to determine the amount of Mpc70 in the strains used for the experiment in A) Mpc70 was tagged with HA. As negative control (neg) a wild type strain was used (YKS32). Samples for immunoblotting were prepared as observed in Fig. 25 B). **C)** Immunoblot analysis of cells used for the experiment in A) during sporulation at 1% KOAc. Cells were taken at the indicated time points after transfer into sporulation medium and subjected to immunoblotting.

The same experiment was performed to check the effect of the high PKA activity induced by bPAC on Spo74. A Spo74-Myc yeast strain manifested the same phenotype with a not real distinguishable phenotype while the control sporulated fine (Figure 27 A). Protein analysis also displayed a drastic reduction of the total quantity of Spo74-Myc compared to the control (Figure 27 B). Western blot of each time point demonstrated in the control a high presence of MP component Spo74-Myc after six and seven hours in KOAc while the amount of proteins for the bPAC strain is almost not visible at these time points (Figure 27 C).

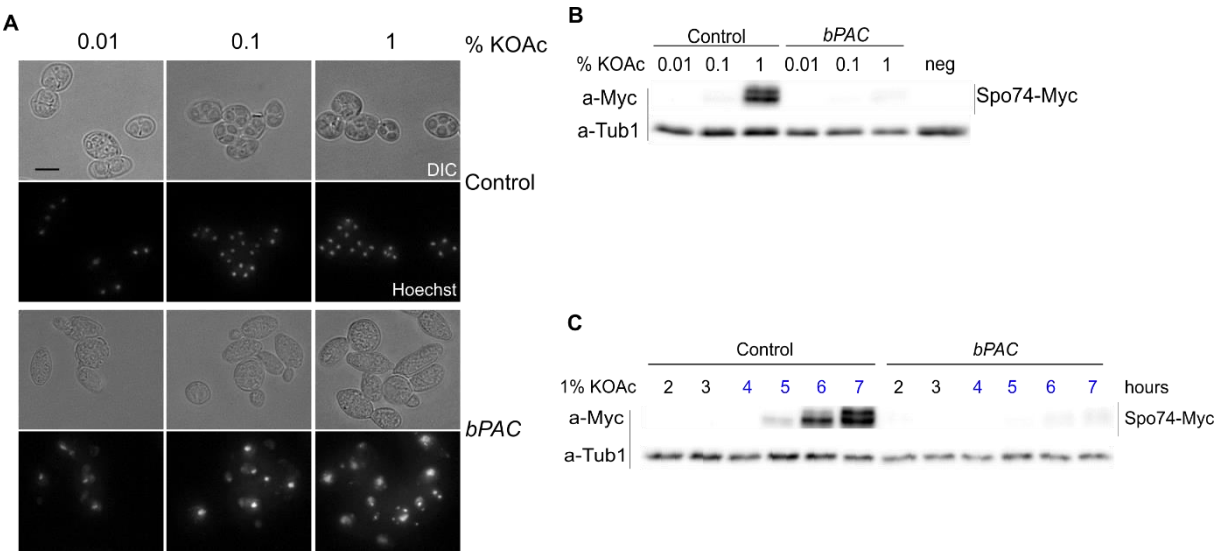

Figure 27: Effect of the high PKA activity induced by bPAC on the abundance of the Spo74-Myc

A) Light and fluorescence microscope analysis of light regulated adenylyl cyclase (YCR561 + pJT4) and control cells (YCR561 + pRS315). Conditions were as described in the legend of Fig. 24 B). **B)** Western blot analysis to determine the amount of Spo74 in the strains used for the experiment in A) Spo74 was tagged with Myc. As negative control (neg) a wild type strain was used (YKS32). Samples for immunoblotting were prepared as observed in Fig. 25 B). **C)** Immunoblot analysis of cells used for the experiment in A) during sporulation at 1% KOAc. Cells were taken at the indicated time points after transfer into sporulation medium and subjected to immunoblotting.

Finally, a strain in which Ady1 protein was tagged with myc antibody was used to test its abundance. Under blue light conditions the bPAC affected meiosis in yeast cells. Control cells sporulated fine (Figure 28 A). In the corrispective western blotting no Ady1-myc was present compared to the total amount of protein of the control (Figure 28 B). All the samples collected

during the time course were singularly loaded on a SDS gel. The signal of Ady1-Myc in the western appeared after the 5th hour in SPO liquid medium 1% while in the strains with expressed bPAC the signal is nearly absent (Figure 28 C).

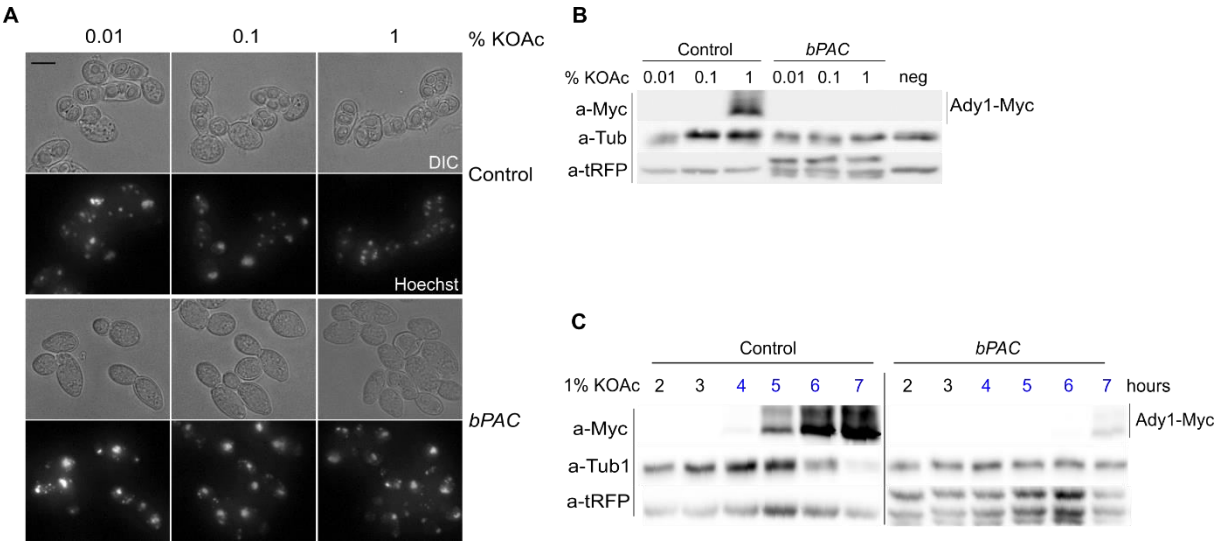

Figure 28: Effect of the high PKA activity induced by bPAC on the abundance of Ady1-Myc

A) Light and fluorescence microscope analysis of light regulated adenylyl cyclase (YDS152 + pDS181) and control cells (YDS152 + pRS316). Conditions were as described in the legend of Fig. 24 B). **B)** Western blot analysis to determine the amount of Ady1 in the strains used for the experiment in A) Ady1 was tagged with Myc. As negative control (neg) a wild type strain was used (YKS32). Samples for immunoblotting were prepared as observed in Fig. 25 B). An antibody specific for tRFP was used to visualize tRFP as control. **C)** Immunoblot analysis of cells used for the experiment in A) during sporulation at 1% KOAc. Cells were taken at the indicated time points after transfer into sporulation medium and subjected to immunoblotting.

A question could be at which phase of meiosis cells are. Therefore, a time course analysis with the strain used in figure 27 A was performed and samples were picked at each time point from the third to the seventh hour in SPO medium 1% (Figure 29 A). The staining showed that control cells entered into meiosis after six hours and at seven hours 50% of the nuclei are dividing. Cells which carry the bPAC showed just a very small fraction of cells in meiosis after 7 hours in SPO medium. (Figure 29 B).

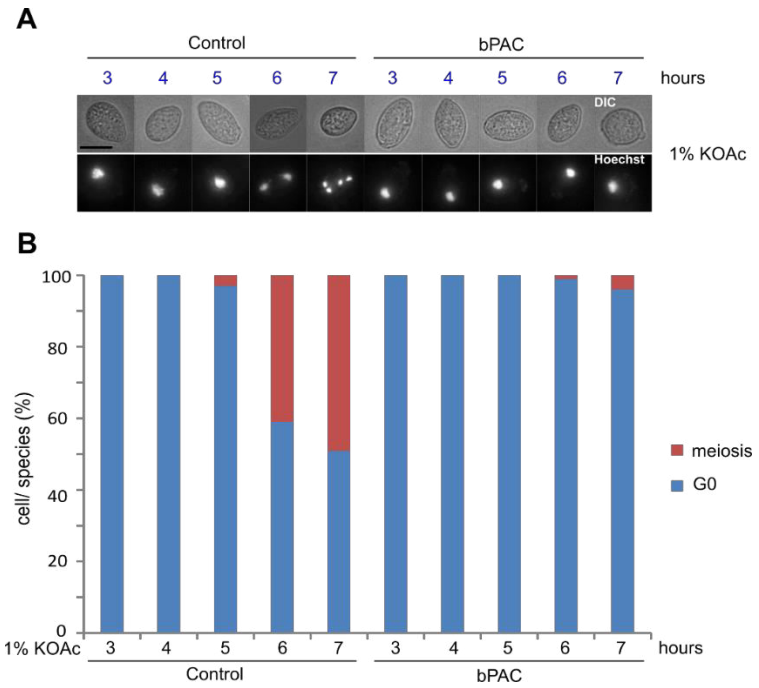

Figure 29: High PKA by bPAC affects entry in meiosis

A) Light and fluorescence microscope analysis of light regulated adenylyl cyclase (YCR484 + pJT4) and control cells (YCR484 + pRS315). Cells during the time course in liquid medium were picked at each time point, stained with Hoechst 33342 and subjected to microscope analysis. Bright field (DIC) and images taken with DAPI fluorescence filter are shown. Bar corresponds to 5 µm. **B)** Quantification of the phenotypes observed from the experiments described in A). Between 100 and 200 cells were assessed for each strain and each condition. Each strain was measured at least three times. In the evaluation were considered the cells with one single DNA stain (G0) and the cells that initiate meiosis.

The assays demonstrated that the bPAC affected extremely the spore formation in *S. cerevisiae* and showed that all the MP components responded to the elevated activity of PKA induced by high cAMP production. However, could be that the absence of the proteins encoded by the middle genes *MPC70*, *SPO74* and *ADY1* are due the fact that the major part of the cells did not reach the meiotic divisions, but the presence in the western of Mpc54 which is encoded by the early gene *MPC54* implicates the arrest of the cells in meiotic prophase. After that the blue light was switched on the protein abundance of Mpc54 decreased at each time points and at every experimental condition in meiosis.

2.1.9 High PKA activity reduces spore formation in an *ady1*Δ mutant

It is known that in absence of the protein Ady1 cells form mostly dyads and monads. Furthermore, the experiment performed during this work showed that many cells did not enter

meiosis or did not form spores. Expression of the dominant active *RAS2*^{G19V} variant examined in an *ady1*Δ strain showed a high number of unsporulated cells as well (Figure 30 A). After that spore formation was induced, sporulation analysis showed a further reduction of the number of spores compared to the control. Only 10 % of the cells sporulated and showed mostly monads with a small presence of dyads at 1% of KOAc (Figure 30 B). An exacerbated reduction of the sporulation efficiency was found at all the concentration of KOAc as well (Figure 30 C).

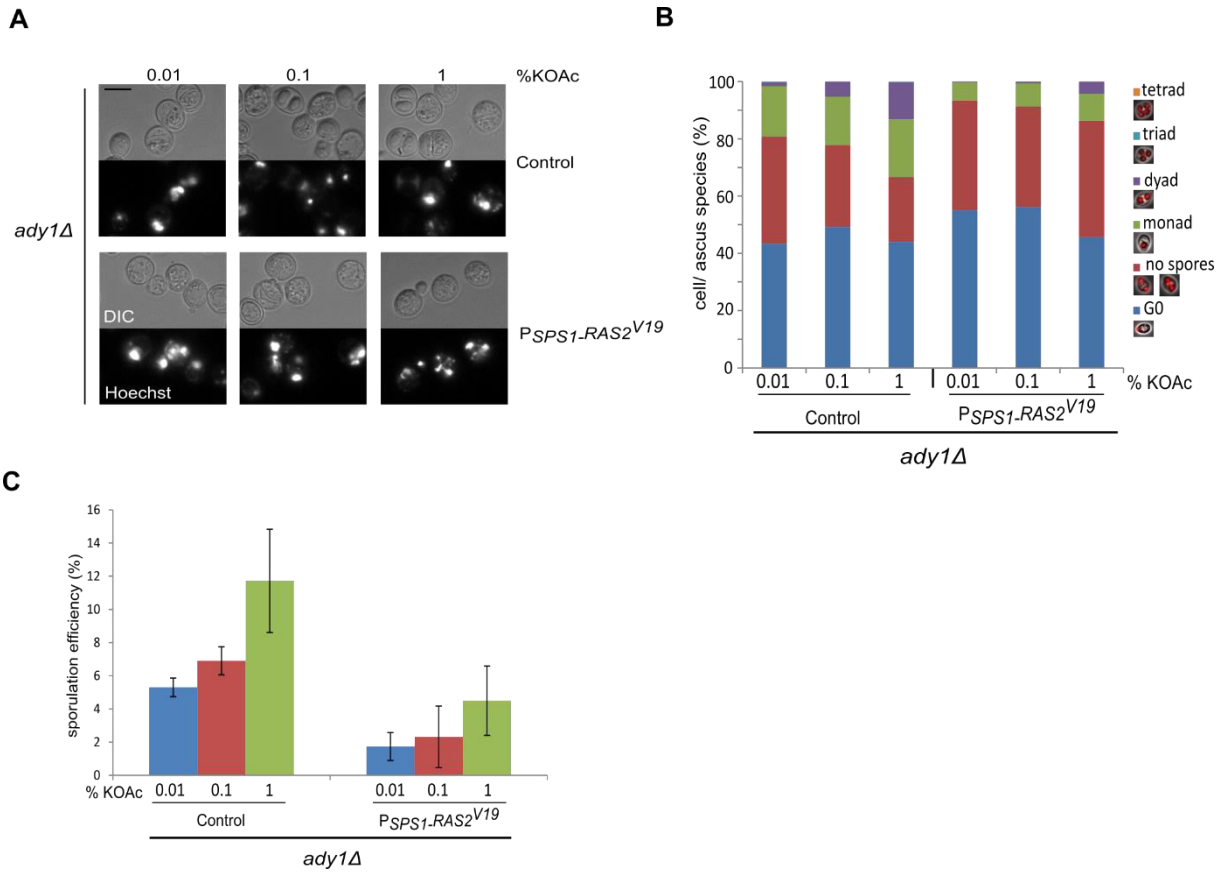

Figure 30: Activation of PKA in an *ady1*Δ mutant reduces spore formation

A) Light and fluorescence microscope analysis of a control strain (YMJ68 + pRS316) and the mutant (YMJ68 + pMJ16). Sporulated cells on plate were stained with Hoechst 33342 and subjected to microscope analysis. Bright field (DIC) and images taken with DAPI fluorescence filter are shown. Bar corresponds to 5 µm. **B)** Quantification of the phenotypes observed from the experiments described in A). Experimental details as described in the legend of Fig. 12 B). **C)** Sporulation efficiency from the strains described in A). Experimental details as described in the legend of Fig. 12 C).

The data shows that hyperactivation of Ras has an effect in a strain in which Ady1 is absent so PKA acts not only through Ady1 but also through other components.

2.1.10: The MP component Spo74 might be a target of PKA

During her bachelor work, the student Ayse Bal investigated the influence of PKA on the Spo74 protein in meiosis. The aim of the study was to inquire whether PKA could affect the sporulation by direct phosphorylation of the Spo74 protein. The consensus sequence of PKA on a protein is Arg/Lys -Arg/Lys - X - Ser/Thr - X, where X is any amino acid. During her work the serine residues of the two consensus sequences for PKA (S253 and S350) have been mutated either individually or both of them to Alanine (A) or Glutamate (E). I tested the effect of the double mutants Spo74^{AA} and Spo74^{EE} on the sporulation. Control cells showed monads and dyads at 0.01% of KOAc, dyads and triads at 0.1% and triads and tetrads at 1% of nutrients. The double mutant Spo74^{AA} showed mostly triads at 0.01% of KOAc and triads and tetrads at 0.1 and 1% of KOAc. The double mutant Spo74^{EE} showed mostly dyads and triads at all concentrations of nutrients (Figure 31 A). As expected sporulation analysis showed that in the control strain the number of tetrads and triads decreased with low concentration of KOAc while the number of dyads or monads increased. In the double mutant Spo74^{AA} however, the number of tetrads or triads was increased at 0.01 and 0.1 % of KOAc while the double mutant Spo74^{EE} showed a decrease of tetrads and an increase of cells in G0 phase at 1% of KOAc plus a slight decrease of the number of tetrads at 0.1% of KOAc. Furthermore, the counting of the cellular types showed that the control strain was regulated by acetate availability while in the two mutants the regulation is lost (Figure 31 B). Subsequently sporulation efficiency was determined. Inhibition of the phosphorylation by double mutant Spo74^{AA} increased the sporulation efficiency at 0.01 and 0.1 % KOAc while using the double mutant Spo74^{EE} which should mimic the phosphorylation, a reduced efficiency at 1% KOAc was found (Figure 31 C).

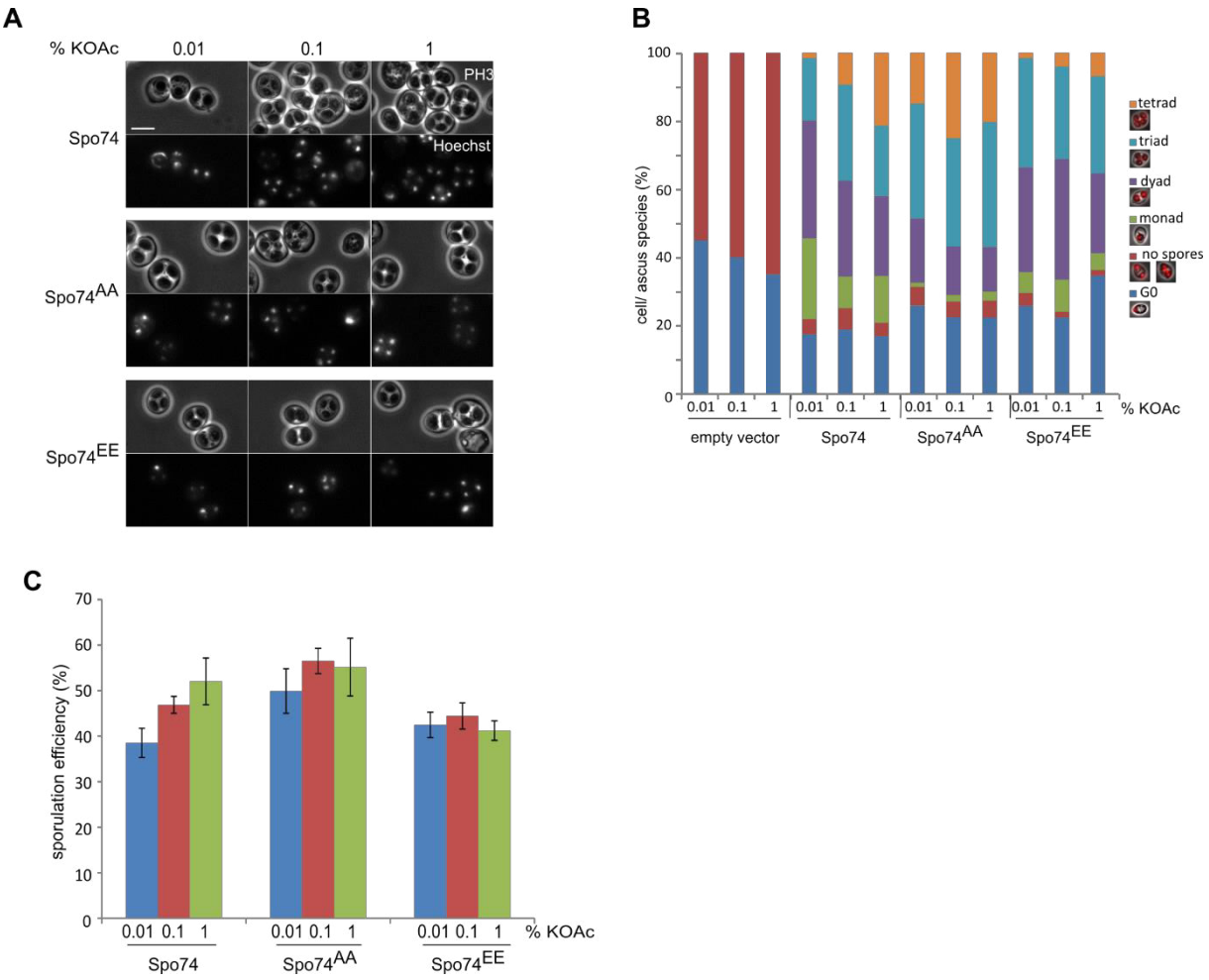

Figure 31: Mutations in the Spo74 PKA consensus sequences change the sporulation behaviour.

A) Light and fluorescence microscope analysis of a control strain (YMK725 + pAB1) and the mutant strains (YMK725 + pAB4 and YMK725 + pAB7). Sporulated cells on plate were stained with Hoechst 33342 and subjected to microscope analysis. Bright field (PH3) and images taken with DAPI fluorescence filter are shown. Bar corresponds to 5 µm. **B)** Quantification of the phenotypes observed from the experiments described in A). Experimental details as described in the legend of Fig. 12 B). **C)** Sporulation efficiency from the strains described in A). Experimental details as described in the legend of Fig. 12 C).

The increased sporulation behavior could be due to the presence of the amino acid alanine in which the OH group is missing therefore phosphorylation is inhibited while the reduced spore formation might be due to the presence of the negative charge of the amino acid glutamate which should mimic the phosphorylation. To check what is the effect of the mutations of the Spo74 consensus sites on the abundance of Spo74 protein I performed a time course analysis with the double mutants in a GFP vector. Control cells showed monads and dyads at 0.01% of KOAc, dyads at 0.1% and dyads and triads with a small fraction of tetrads at 1% of nutrients. The double mutant Spo74^{AA} showed monads and dyads at 0.01% of KOAc and dyads and triads at 0.1 and 1% of KOAc. The double mutant Spo74^{EE} showed mostly monads and dyads

at all concentrations of nutrients (Figure 32 A). All strains sporulated as expected with an increased spore formation and sporulation efficiency at 0.01% of KOAc in yeasts where the PKA consensus sites of Spo74 were modified to Alanine and a decrease of the number of spores and sporulation efficiency at 0.1% and 1% of KOAc in yeast cells where the PKA consensus sites of Spo74 were modified to Glutamate (Figure 32 B, C). Moreover, the presence of a larger number of cells in G0 phase has been observed as well compared to the previous experiment where Spo74 was not tagged with GFP. This decrease of spore formation could be explained by the negative effect of the GFP fusion to Spo74 (Bajgier et al., 2001).

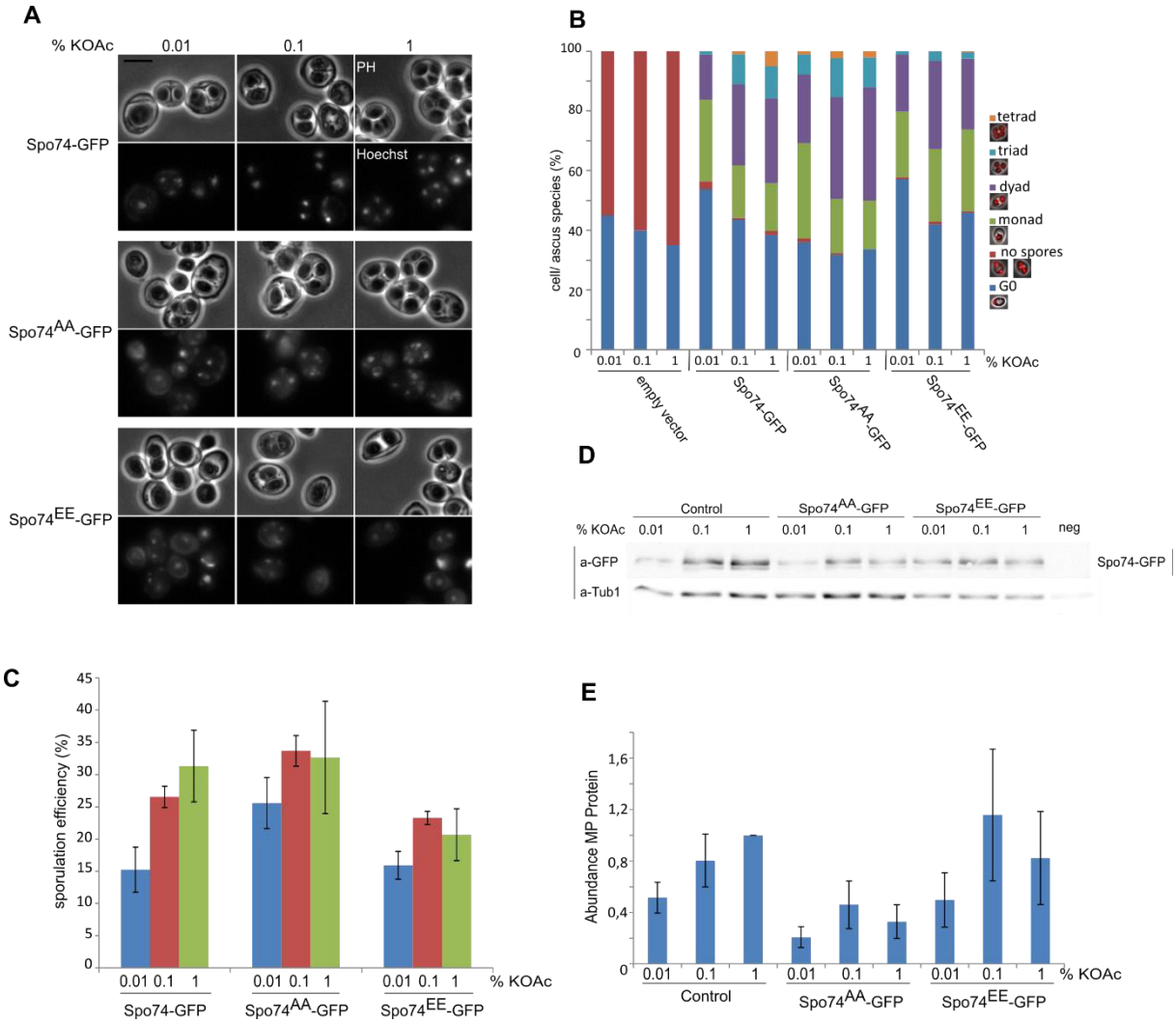

Figure 32: Mutation to alanine in the Spo74 PKA consensus sequence alters the abundance of Spo74 protein.

A) Light and fluorescence microscope analysis of a control strain YMK725 + pMAX2 and the mutant strains (YMK725 + pAB9 and YMK725 + pAB10) performed as described in Fig. 12 A). Bar corresponds to 5 µm. **B)** Quantification of the phenotypes observed from the experiments described in A). Experimental details as described in the legend of Fig. 12 B). **C)** Sporulation efficiency from the strains described in A). Experimental details as described in the legend of Fig. 12 C). **D)** Western blot

analysis to determine the amount of Spo74-GFP, Spo74^{AA}-GFP and Spo74^{EE}-GFP in mutants and control strains showed in A). As negative control (neg) a wild type strain was used (YKS32). Samples for immunoblotting were prepared as observed in Fig. 13 D). An antibody specific for Tub1 was used to visualize Tub1 as loading control **E)** Quantification of the abundance of Spo74-GFP from the western blot in E) normalized to the loading control Tub1. Each strain was measured at least three times. Standard deviations are indicated by error bars.

The western shows a lower presence of Tubulin in the mutant Spo74^{EE} compared to the other two strains (Figure 32 D). Quantification analysis of the protein showed that in the mutant in which the phosphorylation is inhibited, the abundance of Spo74-GFP decreases at all concentrations of nutrients while the mutant which should mimic the phosphorylation did not show any difference compared to the control (Figure 32 E).

2.1.11: Mutated PKA consensus sites in Spo74 do not affect the interaction of Spo74 with the MP components

The previous experiment showed that PKA could affect by direct phosphorylation Spo74. To investigate the effect of the phosphorylation on the protein-protein interactions, a yeast two-hybrid assay can be used. Yeast two-hybrid is an *in vivo* technique that can be utilized to identify interactors of a certain protein of interest (Figure 33). The used assay was the ONPG where the orto nitrophenyl β galactoside ONPG (colorless) is hydrolyzed into galactose (colorless) and orto-nitrophenol (yellow) when β-galactosidase is present.

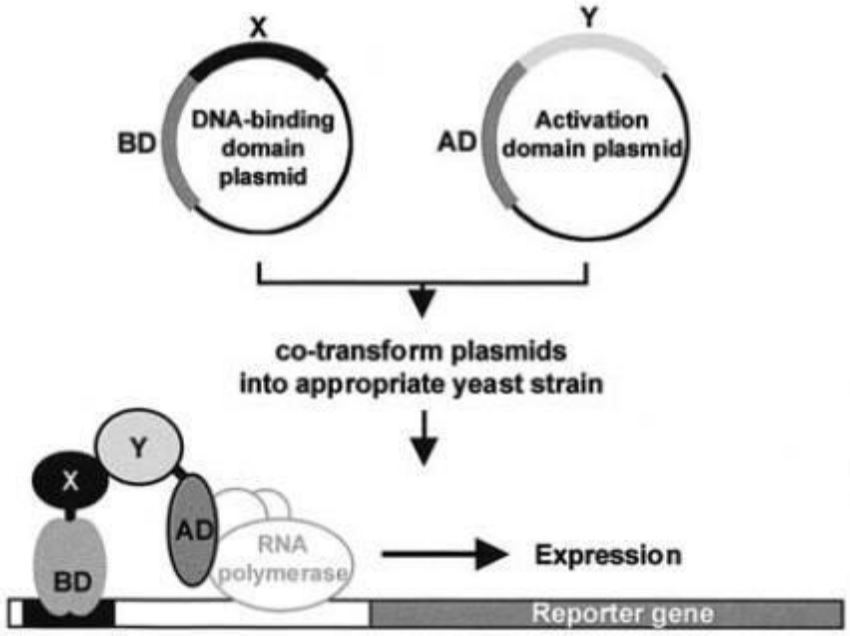

Figure 33: The classical yeast two hybrid system.

A protein of interest is fused to the DNA-binding domain (DBD), and this construct is called bait. Another protein is fused to the transcriptional activation domain (AD) and is called prey. The plasmids

containing the fusion genes encoding for the bait and prey are transformed into the yeast *S. cerevisiae*. The bait binds to the UAS of the promoter and if an interaction occurs between the bait and prey, AD is recruited and a functional transcription factor is reconstituted. This will lead to the recruitment of RNA polymerase and subsequent transcription of a reporter gene, generating a phenotypic signal (figure modified from Causier and Davies 2002).

The Spo74 double mutants have been cloned in yeast two-hybrid vectors by the student Ayse Bal. It is known that Spo74 interacts with itself and with the other MP components Mpc54 and Mpc70 (Nickas et al., 2003). The intent was to see if the mutations effect the interaction of Spo74 with the different proteins of the MP to understand from where the increased and the reduced spore formation due to the modification of the PKA consensus sites come from.

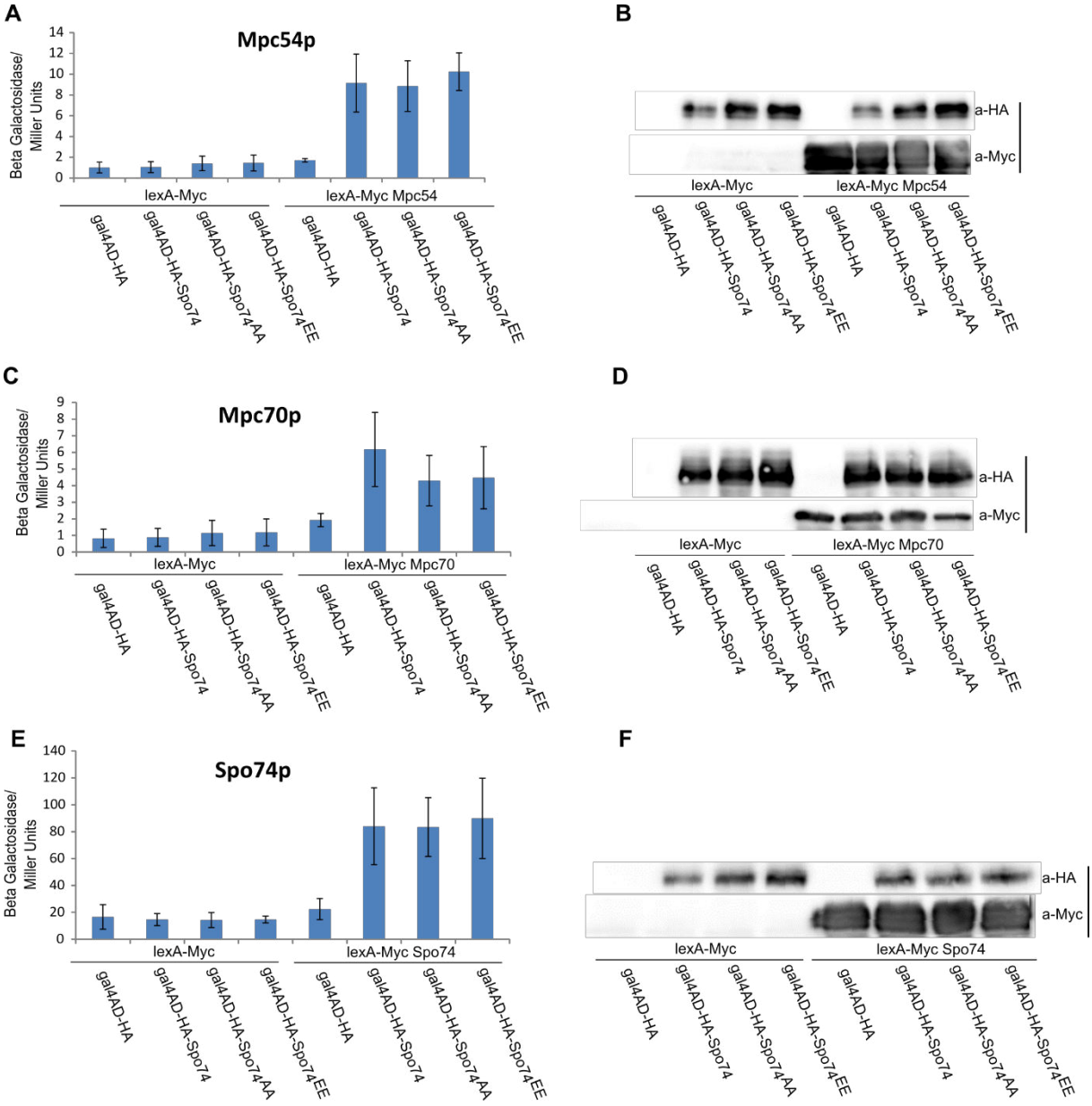

Figure 34: Interactions between Spo74 and the essential MP components is not altered by the modifications in the PKA consensus sites of Spo74

Plasmids carrying the bait and constructed prey were co-introduced into yeast. Transformants of pMM5 and pMM6 alone were used as controls (lexA-myc and gal4AD-HA). The β-galactosidase activity in each transformant was monitored by the liquid ONPG assay and shown in miller units. At least three independent transformants were carried out in ONPG assay. **A)** The interactions between LexA fusion of Mpc54 respectively with GAD fusion of Spo74, GAD fusion of Spo74^{AA} and GAD fusion of Spo74^{EE} are shown. **B)** Western blotting which shows the expression of the constructs in A). **C)** The interactions between LexA fusion of Mpc70 respectively with GAD fusion of Spo74, GAD fusion of Spo74^{AA} and GAD fusion of Spo74^{EE} are shown. **D)** Western blotting which shows the expression of the constructs in C). **E)** The interactions between LexA fusion of Spo74 respectively with GAD fusion of Spo74, GAD fusion of Spo74^{AA} and GAD fusion of Spo74^{EE} are shown. **F)** Western blotting which shows the expression of the constructs in E).

The LexA fusion of the 3 proteins Mpc54, Mpc70 and Spo74 interacted as expected with the GAD fusion of Spo74 and with the two GAD fusions of Spo74 mutants. The ONPG assay performed to analyze quantitatively the interaction between Mpc54 and Spo74 plus the double mutants Spo74^{AA} and Spo74^{EE} did not show any difference by colorimetric analysis (Figure 34 A). The western shows the expression of the constructs used for the two hybrids yeast assay, the LexA binding domain with and without Mpc54 and the transcriptional activation domain AD fused with Spo74, Spo74^{AA} and Spo74^{EE} (Figure 34 B). The interaction between Mpc70 and Spo74, Spo74^{AA} and Spo74^{EE} did not show any difference by ONPG assay (Figure 34 C). The western shows the expression of the LexA binding domain with and without Mpc70 and the transcriptional activation domain AD fused with Spo74, Spo74^{AA} and Spo74^{EE} (Figure 34 D). The interaction between Spo74 and itself plus the two double mutants did not show as well quantitative changes (Figure 34 E). The western shows the respective expressed constructs, LexA with and without Spo74 and the transcriptional activation domain AD fused with Spo74, Spo74^{AA} and Spo74^{EE} (Figure 34 F). With the obtained data seems that the mutated PKA sequences sites do not affect the interaction of Spo74 with all the essential MP proteins. Nevertheless, to confirm this conclusion a two hybrids test between the two Spo74 mutants is requested.

2.2: PKA might regulate spore numbers by using transcription factors

Activated PKA in the Ras/cAMP/PKA pathway has the capacity to regulate several targets by phosphorylation. An important goal to achieve is to know which of such targets are significant for spore formation and spore number control. It has been decided to test specific targets including a set of transcription factors. Transcription factors are proteins that bind to specific DNA sequences, thereby controlling the rate of transcription of genetic information from

DNA to messenger RNA (mRNA). The Ras/cAMP/PKA pathway could as well regulate transcription factors that in turn could activate or repress the expression of three MP components *MPC54*, *MPC70* and *SPO74* leading to a modification of the sporulation phenotype. In fact, it has been shown that overproduction of MP components results in an increased number of spores (Taxis et al., 2005). The goal is to individualize all those transcription factors which can bind to one or more binding sites of the promoter of the MP genes and observe the effect on the sporulation behavior.

2.2.1 Overexpression of transcription factors Nrg1 and Yap1 affects spore formation

In the table 1 transcription factors that have potential binding sites in the promoter of the genes that lead to the formation of the MP proteins, can be seen.

Transcrition factors
AZF1
FKH2
GAT1
GCN4
GIS1
GLN3
GZF3
MSN2
MSN4
NRG1
RTG1
RTG3
SUM1
TEC1
XBP1
YAP1
YRR1

Table 1
Transcription factors which possess binding sequences in at least two MP components promoters

Transcription factors were overexpressed in wild type cells and *ady2*Δ strain. Wild type cells at low concentration of nutrients and *ady2*Δ share the same phenotype, mostly dyads are produced. If one of the transcription factors could regulate the gene expression of the MP components, its overexpression would lead two different results, either the rescue of the phenotype in the *ady2*Δ strain or the production of more spores in the wild type at low concentration of nutrients. Wild type cells were transformed with high copy 2μ plasmids containing the genes encoding for the transcription factors. Control cells showed dyads and triads at 0.01% of KOAc, triads at 0.1% and tetrads at 1% of nutrients. The strain with overexpressed Nrg1 showed mostly diads at 0.01% of KOAc, diads triads and tetrads at 0.1

and triads and tetrads 1% of KOAc. The strain with overexpressed Yap1 showed mostly diads at 0.01% of KOAc, diads triads at 0.1 % and triads and tetrads 1% of KOAc. (Figure 35 A). After sporulation analysis, overexpression of the two transcription factors, Yap1 and Nrg1, displayed less spores within the same ascus and a larger number of unsporulated cells compared the control at all concentrations of KOAc (Figure 35 B). As consequence sporulation efficiency diminished as well (Figure 35 C).

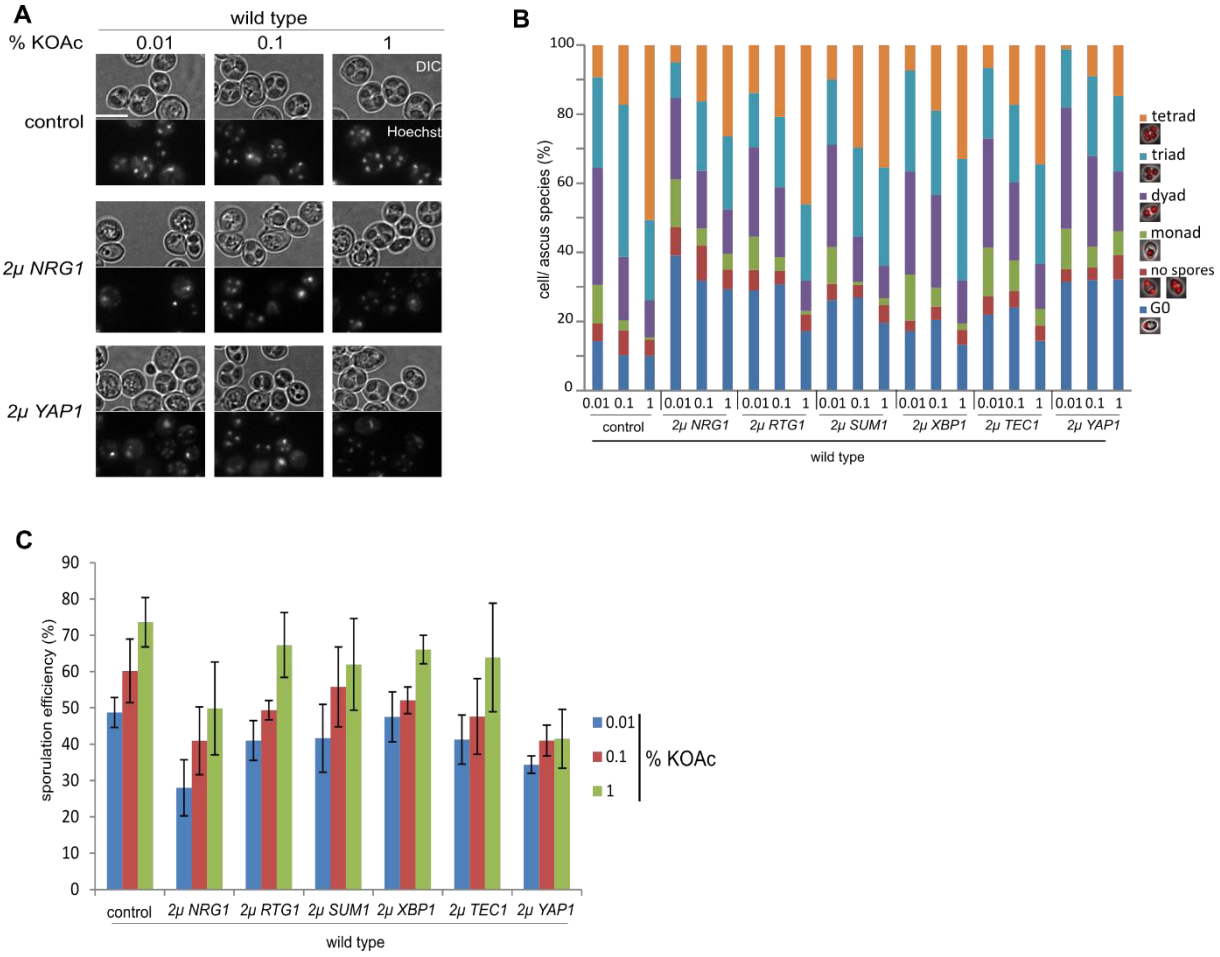

Figure 35: Overexpression in wild type cells of transcription factors with potential binding sites in the promoter of MP genes

A) Light and fluorescence microscope analysis of a control strain YCR77 + pRS315 and the mutants YCR77 + pNRG1 and YCR77 + pYAP1). Conditions were as described in the legend of Fig. 30 A). Bar corresponds to 5 µm. **B)** Quantification of the phenotypes observed from the experiments described in A). Experimental details as described in the legend of Fig. 12 B). **C)** Sporulation efficiency from the strains described in A). Experimental details as described in the legend of Fig. 12 C).

The same set of transcription factors plus the control were transformed in *ady2*Δ mutant. Control cells showed mostly dyads and triads at all concentrations of KOAc with a small fraction of tetrads as well. The strain with overexpressed Nrg1 showed mostly dyads and

triads at all concentrations of KOAc as well. The strain with overexpressed Yap1 showed mostly dyads and triads at 0.01% of KOAc, dyads, triads and tetrads at 0.1 % and 1% of KOAc. (Figure 36 A). Sporulation analysis showed the typical phenotype of such mutant strain, mostly triads and dyads were formed. In figure 36 B is possible to see a high percentage of sporulated cells in the control strain, more than the 90% of the cells sporulated at 0.01 and 0.1 % of KOAc, about 85% of cells sporulated at 1% of nutrients.

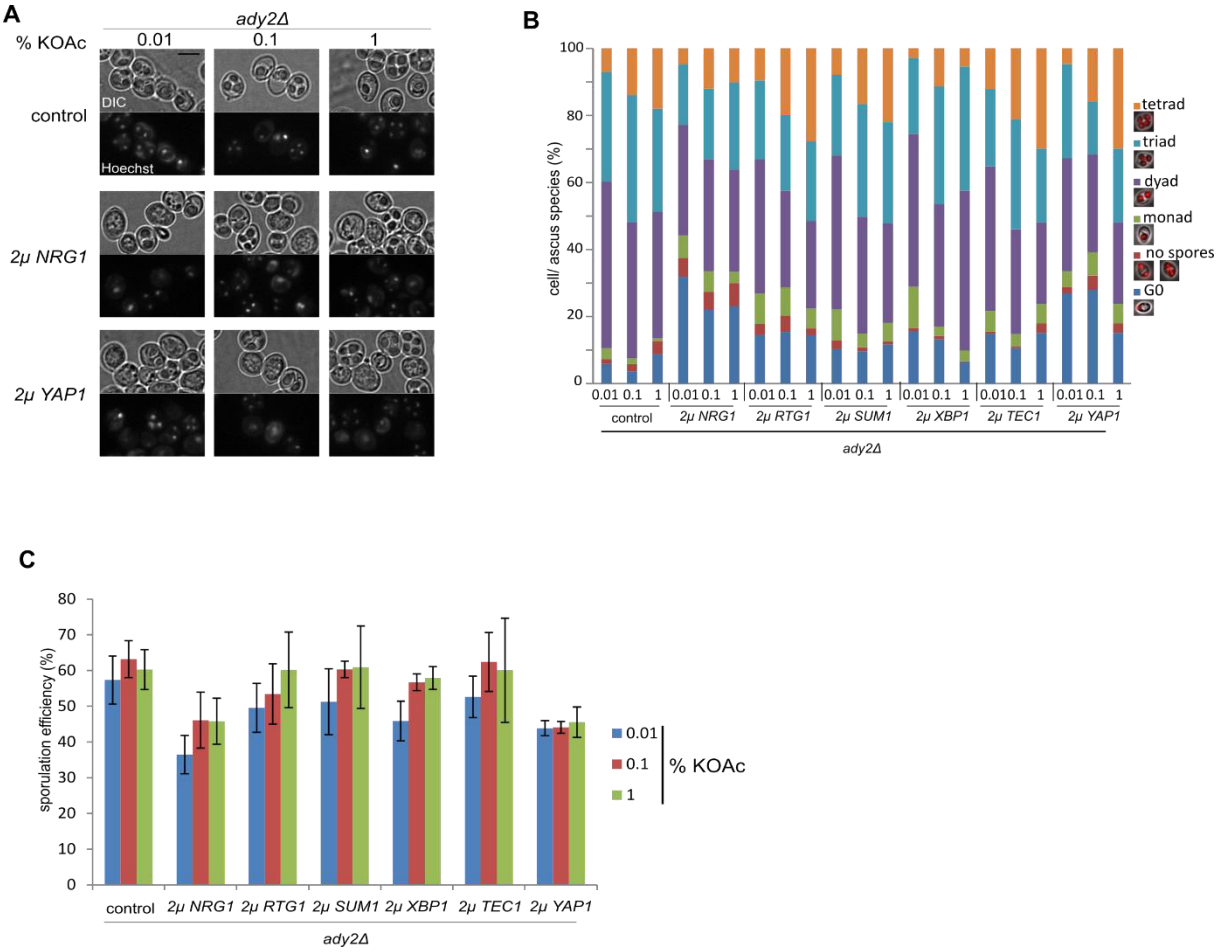

Figure 36: Overexpression in an *ady2*Δ strain of transcription factors with potential binding sites in the promoter of MP genes

A) Light and fluorescence microscope analysis of a control strain YMJ69 + pRS315 and the mutants YMJ69 + pNRG1 and YMJ69 + pYAP1). Conditions were as described in the legend of Fig. 30 A). Bar corresponds to 5 µm. **B)** Quantification of the phenotypes observed from the experiments described in A). Experimental details as described in the legend of Fig. 12 B). **C)** Sporulation efficiency from the strains described in A). Experimental details as described in the legend of Fig. 12 C).

Overexpression of *YAP1* and *NRG1* showed a reduction of the number of spores with triads and dyads. Nevertheless, in this case the percentage of sporulated cells was lower compared the control. Cells with overexpressed *NRG1* showed a number of unsporulated cells between 25% and nearly 40%. Overexpression of *YAP1* led to a percentage of unsporulated cells

between 20 and 35% (Figure 36 B). Sporulation efficiency in the strains with overexpressed Nrg1 and Yap1 decreased compared the control at all concentration of nutrients (Figure 36 C).

2.2.2 Deletion of Nrg1 transcription factor changes spore formation

The overexpression of transcription factors Yap1 and Nrg1 led to a decrease of the rate of the sporulation. The successive step was to delete the respective genes and see how the spore formation is affected. The created *nrg1*Δ and *yap1*Δ strains were subjected to sporulation. Control cells showed mostly dyads at 0.01% of KOAc, dyads and triads at 0.1% and mostly tetrads at 1% of nutrients. *nrg1*Δ showed monads and dyads at 0.01% of KOAc, mostly dyads at 0.1% and triads and tetrads 1% of KOAc. *yap1*Δ showed dyads and triads at 0.01% of KOAc, mostly triads at 0.1 % and triads and tetrads 1% of KOAc. (Figure 37 A). After sporulation analysis *yap1*Δ strain displayed 20 % of tetrads less compared to control at 1 % of KOAc and 15% dyads less at 0.1 % KOAc. The *nrg1*Δ instead showed a larger fraction of dyads at 0.1% of nutrients and about 30 % more monads at 0.01 % of KOAc compared the control (Figure 37 B).

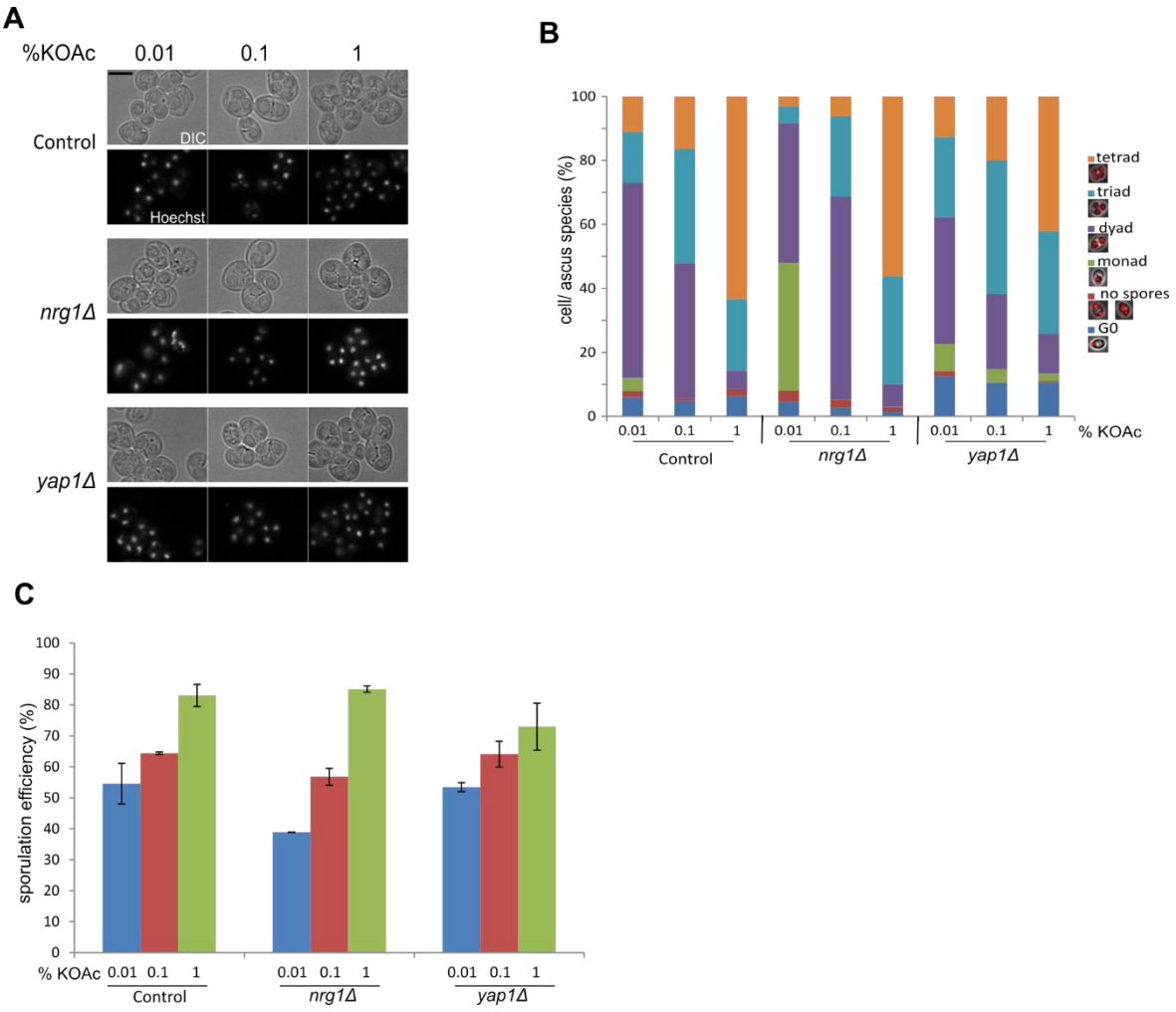

Figure 37: Effect of the deletion of Nrg1 and Yap1 transcription factors on spore formation

A) Light and fluorescence microscope analysis of a control strain YKS32 and the mutants *nrg1*Δ (YMM28) and *yap1*Δ (YMM30). Conditions were as described in the legend of Fig. 30 A). Bar corresponds to 5 µm. **B)** Quantification of the phenotypes observed from the experiments described in A). Experimental details as described in the legend of Fig. 12 B). **C)** Sporulation efficiency from the strains described in A). Experimental details as described in the legend of Fig. 12 C).

Nevertheless, sporulation efficiency for the *yap1*Δ strain did not change compared to the control while as consequence of the reduction of spore formation in the *nrg1*Δ strain the sporulation efficiency at low concentrations of nutrients is lower respect the control (Figure 37 C).

2.3 Overproduction of Ady1 reduces the spore formation in wild type cells

Another part of the investigation was overproducing Ady1 in vegetative growing cells in wild type and in a *ady2*Δ strain in which the ability to form spores is reduced. The goal was to comprehend if the overproduction rescues the phenotype either in the strain in which the ability to form spores is reduced or in the wild type at low concentrations of nutrients. High copy plasmids with *ADY1* were transformed in wild type. Control cells showed dyads and triads at 0.01% of KOAc, triads at 0.1% and triads and tetrads at 1% of nutrients. The strain with overexpressed Ady1 showed mostly dyads at 0.01% of KOAc, dyads and triads at 0.1% and triads and tetrads 1% of KOAc. (Figure 38 A). The analysis showed that the overexpression of *ADY1* affects sporulation just in wild type cells where a higher number of cells in G0 phase are shown at all the percentages of KOAc. Furthermore, a general reduction of the number of spores was found as well (Figure 38 B). As consequence Sporulation efficiency was reduced compared to the control at all concentration of nutrients (Figure 38 C).

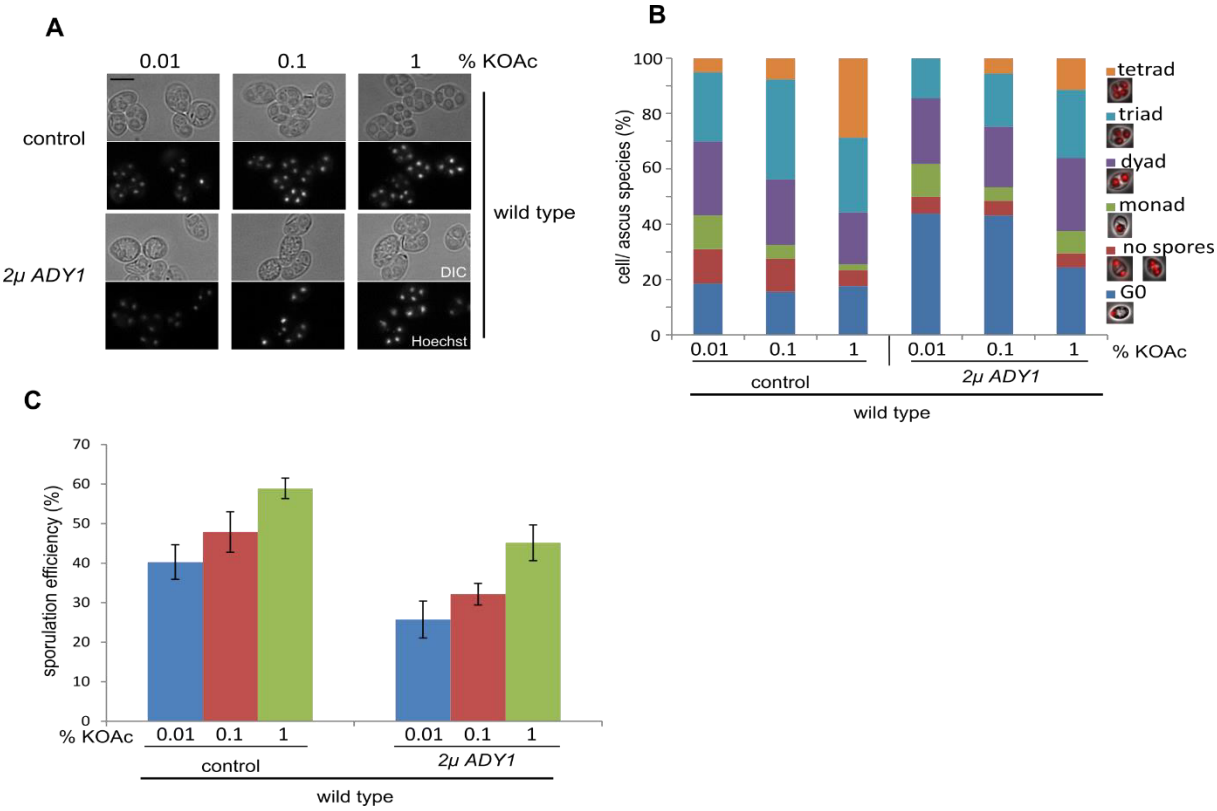

Figure 38: Overexpression of Ady1 in wild type cells affects spore formation

A) Light and fluorescence microscope analysis of a control strain (YCR77 + pRS315) and the mutant (YCR77 + pADY1). Conditions were as described in the legend of Fig. 30 A). Bar corresponds to 5 µm. **B)** Quantification of the phenotypes observed from the experiments described in A). Experimental details as described in the legend of Fig. 12 B. **C)** Sporulation efficiency from the strains described in A). Experimental details as described in the legend of Fig. 12 C).

High copy plasmids with *ADY1* were transformed in *ady2*Δ. Control cells showed mostly dyads and triads at all concentrations of KOAc with a small fraction of tetrads as well. The strain with overexpressed Ady1 showed the same phenotype. (Figure 39 A). Therefore, concerning the overproduction of Ady1 in *ady2*Δ the sporulation analysis did not display any difference compared the control, no changes in the spore formation and in the sporulation efficiency were shown (Figure 39 B, C).

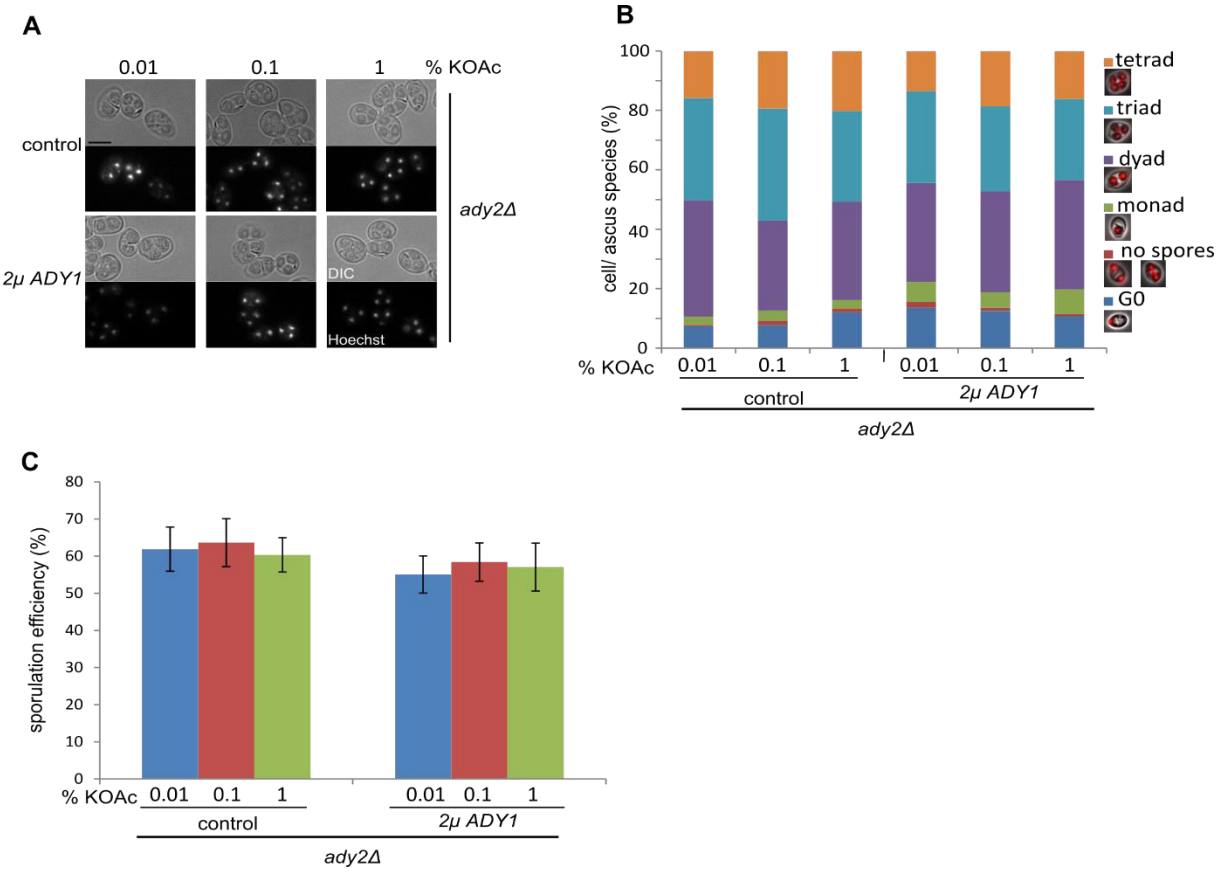

Figure 39: Overexpression of Ady1 in an *ady2*Δ strain does not influence spore formation

Light and fluorescence microscope analysis of a control strain (YMJ69 + pRS315) and the mutant (YMJ69 + pADY1). Conditions were as described in the legend of Fig. 30 A). Bar corresponds to 5 µm. **B)** Quantification of the phenotypes observed from the experiments described in A). Experimental details as described in the legend of Fig. 12 B. **C)** Sporulation efficiency from the strains described in A). Experimental details as described in the legend of Fig. 12 C).

I conclude that overproduction of Ady1 affected negatively merely spore formation in wild type strain and not in *ady2*Δ.

2.4 Deletion of *FOB1*, *PNC1* and *SNF4* genes effects sporulation

The main signaling pathway activated by glucose in yeast is the Protein Kinase A (PKA) pathway. It is responsible together with other signaling pathways of the initiation, maintaining and completion of the gametes developmental program in *Saccharomyces cerevisiae*, but it is unknown if many targets of PKA present in other pathways are involved in the sporulation process. Three possible targets are Pnc1, Fob1 and Snf4 which have been tested for spore formation and sporulation efficiency. Control cells showed monads and dyads at 0.01% of KOAc, dyads and triads at 0.1% and triads and tetrads at 1% of nutrients. *fob1*Δ showed monads and dyads at 0.01% of KOAc, dyads and triads at 0.1% and triads and tetrads 1% of

KOAc. *pnc1*Δ showed dyads and triads at 0.01% of KOAc, triads and tetrads at 0.1% and mostly tetrads 1% of KOAc. *snf4*Δ did not show spores at all concentrations of nutrients. (Figure 40 A).

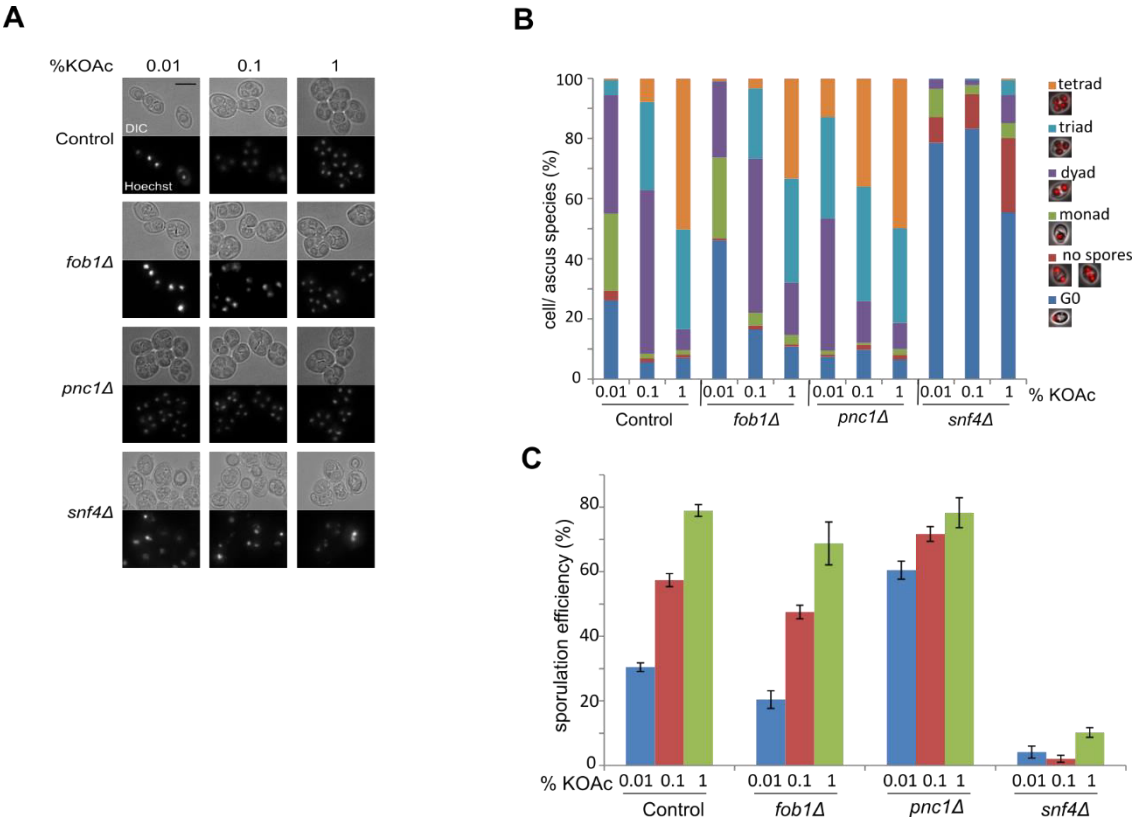

Figure 40: Absence of *FOB1*, *PNC1* and *SNF4* genes changes spore formation

A) Light and fluorescence microscope analysis of a control strain (YKS32) and three different strains in which *FOB1*, *PNC1* and *SNF4* have been deleted (YMM45, YMM52 and YMM55). Conditions were as described in the legend of Fig. 30 A). Bar corresponds to 5 µm. **B)** Quantification of the phenotypes observed from the experiments described in A). Experimental details as described in the legend of Fig. 12 B). Sporulation efficiency from the strains described in A). Experimental details as described in the legend of Fig. 12 C).

Deletion analysis of *PNC1* displayed an increase of the spore formation at 0.01% and 0.1% of potassium acetate, in fact the number of triads and tetrads in the null strain is higher compared the control. Deletion of the regulatory subunit of Snf1 called Snf4 led to a dramatic drop of the spore formation at all concentrations of nutrients while the absence of Fob1 affected negatively the sporulation as well with less tetrads formed at 0.1 and 1% of KOAc and more G0 phase cells at 0.01% of KOAc (Figure 40 B). This had a repercussion on the sporulation efficiency, the *pnc1*Δ showed a higher value at 0.01 and 0.1 % of nutrients, *fob1*Δ showed a

lower value at all concentrations of nutrients compared to the control while *snf1*Δ sporulation efficiency was drastically reduced (Figure 40 C).

3. Discussion

3.1 PKA controls the essential MP components plus the protein Ady1

This work has confirmed the previous knowledge about the cAMP/PKA pathway, high PKA has a negative effect on the spore formation. Exactly here it has been studied how PKA controls spore formation. Furthermore, part of this project wanted to answer the question if Ady1 links PKA to the spore formation. A lot is known about the role of PKA on the entry in meiosis. The initiation of meiosis is a process controlled by nutrients. Glucose and the non-fermentable carbon source acetate for example are crucial for the meiotic entry because they are responsible of the regulation of signaling pathways which lead to the activation of the master initiator of meiosis Ime1 (Piekarska et al., 2010). When glucose is absent PKA is inactive, therefore the repressor Sok2 is not activated while the two activators Msn2/Msn4 are in the nucleus and can bind the activator binding site IREu (Alepuz et al., 1998). The acetate instead when converted in CO_2 leads to the alkalization of the medium (Hayashi et al., 1998a; Ohkuni et al., 1998). This raised pH causes the activation of the Rim101 signaling pathway which through the transcription factor Rim101 alleviates the repressive activity of Smp1 that has the capacity to bind the binding site UASrm (Su and Mitchell, 1993). When Ime1 then is active together with Ume6 the transcription of *IME2* occurs (Piekarska et al., 2010). *IME2* encodes for the kinase Ime2 that together with Ime1 is responsible for the activation of all the early genes. Furthermore, Ime2 triggers the transcription of the middle gene *NDT80* which encodes for a transcription factor that is the responsible of the expression of all those genes that are required for the prophase I to metaphase I transition, for the meiotic divisions and for the initiation of spore morphogenesis. Studies have shown that cAMP levels rise during meiosis I and meiosis II and decrease toward the end of the spore formation (Uno et al., 1985) suggesting that PKA is still active after the entry in meiosis. Recently it has been shown that PKA affects spore formation in a period comprised the commitment to meiosis and the initiation of spore formation, exactly between the end of prophase and the beginning of metaphase II (Jungbluth et al., 2012). In the same work Jungbluth et al. showed that this kinase could regulate the activity of the MP components since the PKA target Ady1, a protein present in meiosis II is necessary for the localization of Spo74 and Mpc70 to the SPBs together with the already known Mpc54 (Deng and Saunders, 2001). In 2005 Taxis et al., already described that different amounts of MP proteins have an influence on the regulation of the number of spores. Here it has been demonstrated that the essential MP components and

the protein Ady1 reacted to modifications of the PKA activity. The kinase regulates the sporulation process after commitment to meiosis by affecting the MP components plus Ady1. Mutants in the Ras/cAMP/PKA pathway which lead to a high activity of PKA showed a decrease in the number of spores in *Saccharomyces cerevisiae* (Jungbluth et al., 2012). High PKA activity by bPAC affected growth and meiotic entry (Trauth bachelor thesis, 2016). In my work, it has been studied what occurs at the level of the MP and other components like Ady1 when high PKA activity by bPAC prevents the sporulation. Microscope pictures showed that is very difficult to understand in which specific phase cells are. The analysis of the total amount of Mpc54 in all phases of meiosis showed a decrease of its abundance compared to the control while Mpc70, Spo74 and Ady1 proteins were almost absent. In this way is difficult to understand if and how the proteins are affected by high PKA therefore the analysis of the proteins at specific time points during the course of sporulation was performed. After two hours that sporulation was induced Mpc54 was already present in the strain with expressed bPAC. *MPC54* is an early gene and is expressed in the initial phase of meiosis. Thus, this approach could indicate that cells are in prophase. Furthermore, the reduction of the abundance of Mpc54 in the bPAC strain seen after usage of the blue light could imply an effect of PKA on Mpc54 in this specific phase. That is really important because Jungbluth in the previous study found out that PKA affects sporulation in the period comprised the end of prophase and the beginning of metaphase II. Sporulation in *Saccharomyces cerevisiae* is characterized by a point of transition important for the differentiation of the process. This commitment to meiosis occurs between the end of prophase and the beginning of meiosis I (Simchen 2009). Analysis of the gene-expression program of committed cells suggested that this point is not ensured by stabilization of the process but by an active modulation of the gene-expression program of the cells (Friedlander et al 2006). The study of Mpc70, Spo74 and Ady1 revealed that these components are not visible on a SDS page compared to the controls when bPAC is expressed. *MPC70, SPO74* and *ADY1* belong to the family of the middle genes therefore expressed in the middle phase of meiosis. With this approach was demonstrated that high PKA activity by bPAC arrests the spore formation before the meiotic divisions and most likely influences the expression of the middle genes. In the end cells do not reach the point at the onset of meiosis II in which the MP and the component Ady1 form. Mpc70, Spo74 and Ady1 resulted then absent. Mutants in the Ras/cAMP/PKA pathway which lead to a low activity of PKA showed an increased spore formation (Jungbluth et al., 2012). Furthermore, in the same article the lowered PKA activity by mutation of the adenylyl cyclase $cyr1^{K1712A}$ led to a higher number of spores at low concentration of KOAc. In my work, the

same reduction of the PKA activity showed an increase of the abundance of Mpc70, Spo74 and of the protein Ady1 which are encoded by middle genes. This is a clear indication that cells have passed the commitment to meiosis and are doing the meiotic divisions suggesting us that PKA might influence spore formation during all stages of meiosis. Overall my data revealed that PKA affects spore formation by influencing the abundance of all the essential MP components Mpc54, Mpc70, Spo74 plus Ady1. Could be that PKA has an effect at the level of the synthesis and at the level of the translation. In fact, PKA has been identified as an important regulator of gene-specific translation activity (Vaidyanathan et al., 2014). A possible explanation is that PKA could affect the MP components and Ady1 at a transcriptional level by influencing the expression of the genes *IME1* and *NDT80,* the first is responsible of the expression of the early genes included *MPC54*, the latter responsible of the expression of the middle genes included *MPC70, SPO74* and *ADY1*. Furthermore, the experiment performed with the two mutations on the PKA consensus sites in Spo74 displayed two different phenotypes compared to the normal sporulation behavior. This would demonstrate that PKA could regulate the MP component Spo74 directly at a post translational level by phosphorylation and at the same time could have a direct effect on the MP through this protein. This ulterior experiment reinforces the idea that PKA affects also the formation of the MP in the middle phase of spore formation like in meiosis II, therefore after commitment to meiosis. Jungbluth et al. in 2012 performed an experiment to see what is the effect on the meiotic divisions in a strain in which Ras proteins were depleted. Unlike my kind of experiment, he checked the meiotic events, therefore the mono, bi or tetranucleated cells during sporulation. He could demonstrate that meiotic divisions were delayed at low acetate concentrations in both control and Ras depleted cells. This showed that acetate availability, but not Ras activity, influenced the timing of the meiotic divisions. In my experiments, the $cyr1^{K1712A}$ mutation showed that cells enter in meiosis earlier compared the control regardless on the nutritional conditions. Furthermore, at low concentration of KOAc the number of cells in meiosis is higher in the mutant. This could be because the reduced activity of Cyr1 and therefore the reduction of the production of cAMP might affect the timing of the regulation of PKA, thus the downstream target Ime1 responsible of the initiation of meiosis is activated in advance. As result all the regulatory cascade which controls the events of sporulation is activated earlier. The kinase influences the different components directly and indirectly. The sporulation specific factor Ady1 that is phosphorylated by PKA *in vitro* (Ptacek et al., 2005) in my work is shown that it could be indirectly regulated by PKA *in vivo*. My data suggest that PKA has an indirect effect on the MP by affecting Ady1 during the

second meiotic division, thus when Ady1 is formed and can localize all the MP components to the SPB. How Ady1 affects the MP can be just speculated. Ady1 has a physical interaction with Hrr25 kinase (Petronczki et al., 2006). Could be that Ady1 controls the activity of this kinase or recruits its substrates. Hrr25 kinase could be involved in the activation of the spindle pole or the formation of the MP by phosphorylating the SPB components. In fact, recently it has been found that the recruitment of Mpc70 to SPBs requires Hrr25 activity (Argüello-Miranda et al., 2017). Overexpression of Ady1 led to a reduction of the spore formation in wild type cells while in *ady2*Δ mutant, a strain in which the capacity to form spore is decreased, the spore formation was not affected. Could be that the presence of high amount of Ady1 protein may form heterodimers between Ady1 and an eventual component present in meiosis II which would disturb the spore formation. This could be Spo13 protein. *SPO13* gene is expressed uniquely in meiosis (Buckingham et al. 1990) and regulates the progression of meiotic nuclear divisions in *Saccharomyces cerevisiae* (McCarrol and Esposito, 1994). A yeast two-hybrid assay showed an interaction between Ady1 and Spo13 (Uetz et al., 2000). Furthermore, it has been shown that in absence of Spo13, spore formation is decreased (Enyenihi and Saunders 2003). The formation of heterodimers with Ady1 would impede Spo13 to play its role during sporulation. A possible experiment to test this model might be to overproduce Spo13 and see the sporulation behavior. Another reason might be that the eventual formation of heterodimers with Hrr25 would affect the interaction with Mpc70. As consequence Mpc70 recruitment to the SPB could be prevented. Ady1 was shown to be localized partially in the nucleus in meiosis (Deng and Saunders, 2004). In the present work, it has been found that in sporulating cells Ady1 is present in the nucleus either in cells with reduced PKA or in control cells. Overall the experiments with Ady1 confirmed the involvement of this protein in the spore formation. The fact that Ady1 is a meiotic protein reinforced the assessment that PKA is active after commitment to meiosis and exactly even in meiosis II. All the essential MP components plus the protein Ady1 resulted targets of PKA. PKA affects directly the MP formation by affecting Spo74 and it has an indirect effect by Ady1. Moreover, high PKA by expressed bPAC affected the MP, it inhibits its formation due to an early activation of the kinase. This has a dramatic influence on the spore formation, indeed as consequence sporulation is prevented. With my tests, I found out that PKA affects spore formation at the level of the MP formation which takes place during meiosis. The performed experiments indicated that PKA affects the generation and the abundance of the essential MP components. Ady1 which has the role to localize the MP proteins to the SPB is by itself affected by PKA as well. I can conclude that PKA has an influence on spore

formation after commitment to meiosis by affecting the abundance of meiotic plaque components and their ability to form a MP at SPBs. The experiment with the bPAC revealed also the powerful and the advantages of this optogenetic tool compared to the classical genetic approaches. The experiment with Mpc54 showed that the activation of bPAC is very fast and has an immediate effect on the abundance of the protein. This is very advantageous because it requires just the exposure of the yeast cells to blue light and involves only the use of a single plasmid instead of more demanding chromosomal manipulations.

3.2 PKA regulates the activity of different components

It is well known that PKA has numerous targets and controls many processes in response to nutrients. The data obtained from this work revealed that components of other pathways with connections to PKA influence sporulation or spore number control.

3.2.1 PKA controls the NAD+ salvage pathway

NAD is involved in redox reactions and it is an essential cofactor used in many biological processes like longevity, life span extension and transcriptional regulation (Lin and Guarente, 2003). It could be indeed that the regulation of the transcriptional regulation necessary for activating and coordinating spore formation is affected by the absence of *PNC1*. This would lead to a loss of the regulation. The Ras/cAMP/PKA pathway regulates carbohydrate metabolism and influences the mitochondrial respiratory capacity. The pathway exerts during vegetative growth and during sporulation a negative impact on the glyoxylate cycle and on the TCA cycle affecting the amount and the activity of certain enzymes (Hedbecker et al. 2004; Ordiz et al. 1996; Ptacek et al. 2005, Roosen et al. 2005; Schneper et al. 2004; Swieger et al. 2006; Zaman et al. 2008; Jungbluth et al. 2012). It could be that the reduction of the NAD+ by deletion of the Pnc1 affects the glyoxylate cycle at the level of the reaction malate-oxoloacetic acid which requires NAD. In this way, the acetil group from the acetil-CoA cannot bind the oxoloacetic acid with the formation of citrate.

3.2.2 PKA is connected to spore formation through the ribosome biogenesis

PKA controls growth by controlling the expression of ribosomal protein genes and ribosome biogenesis genes. Fob1 is involved in rRNA production because it blocks the replication fork barrier RFB. In its absence, the RFB is not bound and blocked, hence, in the S phase of the

cell cycle when replication starts the replicative fork continues its undisturbed progression in the direction of 35S rRNA transcription. Therefore, when Fob1 is missing, it could be that collisions between replication and transcription machineries take place with the consequent disturb in the production of rRNA. The ability of a cell to properly regulate translation in part depends on the rate of ribosome biogenesis and the presence of less ribosomes due to the absence of Fob1 could be associated to the decrease of the number of spore (Steffen, et al. 2008). Fob1 regulates the rDNA recombination (Kaeberlein et al. 1999) and PKA affects by Rrn3 the RNA polymerase I which transcribes for the rDNA (Howard et al., 2003). The different sporulation behavior found by deletion of *FOB1* would connect ribosome biogenesis to spore formation.

3.2.3 PKA controls the metabolism of alternative carbon sources

PKA affects negatively the Snf1 pathway by phosphorylating the Snf1-activating kinase Sak1 (Barret at al., 2012). Deletion of *SNF4* led to a dramatic reduction of the spore formation. That could be because Snf4 cannot activate the kinase Snf1, the major component of the glucose repression pathway which has an important role in the expression of the genes involved in the metabolism of alternative carbon sources. It might be indeed that in absence of Snf4 the catalytic heterotrimeric complex cannot form, therefore active Snf1 with the subunit Gal83 cannot enter in the nucleus to lead derepression of Mig1 repressor. The expression of genes involved in the metabolism of alternative carbon sources cannot occur. To test this assumption would be useful to check if the mutant grows in a medium in which is present an alternative carbon source like for example acetate (YPAc). Nevertheless, it was shown that *snf4* null mutants are viable but are unable to grow on maltose or on non-fermentable carbon sources (Neigeborn and Carlson, 1984; Schuller and Entian, 1988; Entian and Zimmermann, 1982). In absence of Snf4, the regulation of Snf1 does not occur so it is inactive and not able to lead to the expression of *IME1* and *IME2* responsible of the initiation of meiosis. This mechanism could explain well the observed phenotype.

3.2.4 Inactivation of Nrg1 reduces spore formation

The transcription factor Nrg1 is the repressor of the STRESS genes. *Saccharomyces cerevisiae* is able to adapt to stresses due environmental pH conditions by altering the expression of the STRESS-responsive genes. The gene expression alteration is transient

(Causton et al., 2001; Gasch et al., 2000). Cells adapt to the changed environment, their gene expression resets to a program close to that observed in the absence of stress. During sporulation, due to the presence of acetate, the environmental pH conditions led to the adaption of the cell by changing its genomic expression. But it could be that in absence of the repressor Nrg1, the adaption does not occur properly, therefore there is a decrease of spore formation at low concentration of KOAc. This is in contrast with the experiment performed with a reduced cAMP where at low concentration of nutrients there is an increase of the number of spores. The overexpression of the repressor *NRG1* showed a decrease of the spore formation as well. In normal conditions could be that Nrg1 forms a complex with other two different partners. In presence of high copies of Nrg1 instead could be that such complex is inactive because Nrg1 binds separately the other two components, therefore Nrg1 by overexpression would result inactive as well. Another explanation could be that high copies Nrg1 could affects downstream targets. Nrg1 indeed is a downstream of the Rim101 pathway. Could be that in presence of high Nrg1 the transcription factor Rim101 cannot repress it therefore Nrg1 inhibits the stress responsive genes. Furthermore, it is a downstream of the Snf1 pathway. In response to glucose limitation the Snf1 kinase relieves Nrg1-mediated repression of the glucose repressed genes but when Nrg1 is overexpressed could be that the kinase cannot repress it. Nrg1 could also affect sporulation by affecting with its repressive role the middle to late sporulation genes (Rothfels et al., 2005). When Nrg1 is present at high levels in the cell it could bind the promoter of such genes during the middle-late phase of spore formation and could influence for example spore wall assembly and the enclosure of the prospore membrane. Nrg1 could control early and middle-late phase in meiosis.

3.3 Bicarbonate in liquid medium does not increase spore formation

The experiment executed to see how the spore formation is regulated in presence of bicarbonate in liquid SPO medium did not show any difference compared to the control in absence of bicarbonate. Proliferating cells respiring acetate in presence of extracellular bicarbonate which alkalizes the medium are inhibited for cell growth and multiplication. Cells which arrested the division cycle at the G1 phase entered the G0 stationary phase. This alkalization stimulates meiosis and sporulation (Hayashi et al. 1998a). Furthermore, *S. cerevisiae* produces an extracellular factor MEP required for efficient meiosis and sporulation (Hayashi et al. 1998b). In 2012 Jungbluth et al. found out that intracellular carbon dioxide/bicarbonate levels as well are important for sporulation. Either in the control strain or

in strains lacking Ady2 and Nce103 the presence of bicarbonate raised the number of spores. The actual work showed a different result compared the previous knowledge. This different result is due to different working conditions. Jungbluth in 2012 placed solid sodium hydrogen carbonate crystals in the lid of a SPO-plate and moistened it with water to create a CO_2-enriched atmosphere. In the present work sporulation occurred in liquid medium where bicarbonate in solution was added. Could be that hydrogen carbonate ion HCO_3^- cannot pass the plasma membrane by diffusion as the hydrophobic gas molecule CO_2 can do. In fact, hydrogencarbonate ion is a charged and not a small polar molecule, therefore it is repelled by the hydrophobic interior of the bilayer. Intracellular bicarbonate then cannot rise and as consequence sporulation cannot enhance as well.

3.4 bPAC activity affects plasma membrane localization of Ras

Previously, Ras has been found localized in vegetative cells at the cell periphery and in the nucleus (Leadsham et al., 2009). The same nuclear and peripheral signal was found in vegetative cell and slightly reduced in sporulating cells (Jungblut et al., 2012). Yet, during the same work, it was shown that acetate does not modulate Ras activity. Nevertheless, an increased high PKA activity by expression of the dominant active $RAS2^{G19V}$ variant led to a higher number of cells showing Ras localized in the cell periphery indicating higher Ras activity due to the usage of the hyperactive $RAS2^{G19V}$ allele. The experiment performed in the actual work where the photoactivated adenylyl cyclase bPAC has been used to increase the PKA activity showed that the number of cells in which Ras is localized in the plasma membrane decreased. The two experiments are completely different but with the same intention, increasing the PKA activity.

3.5 Proposed models for the regulation of spore formation by PKA

Through the obtained data a model which explains how PKA regulates targets can be set. In Figure 40 is possible to see how PKA controls the MP components and the protein Ady1.

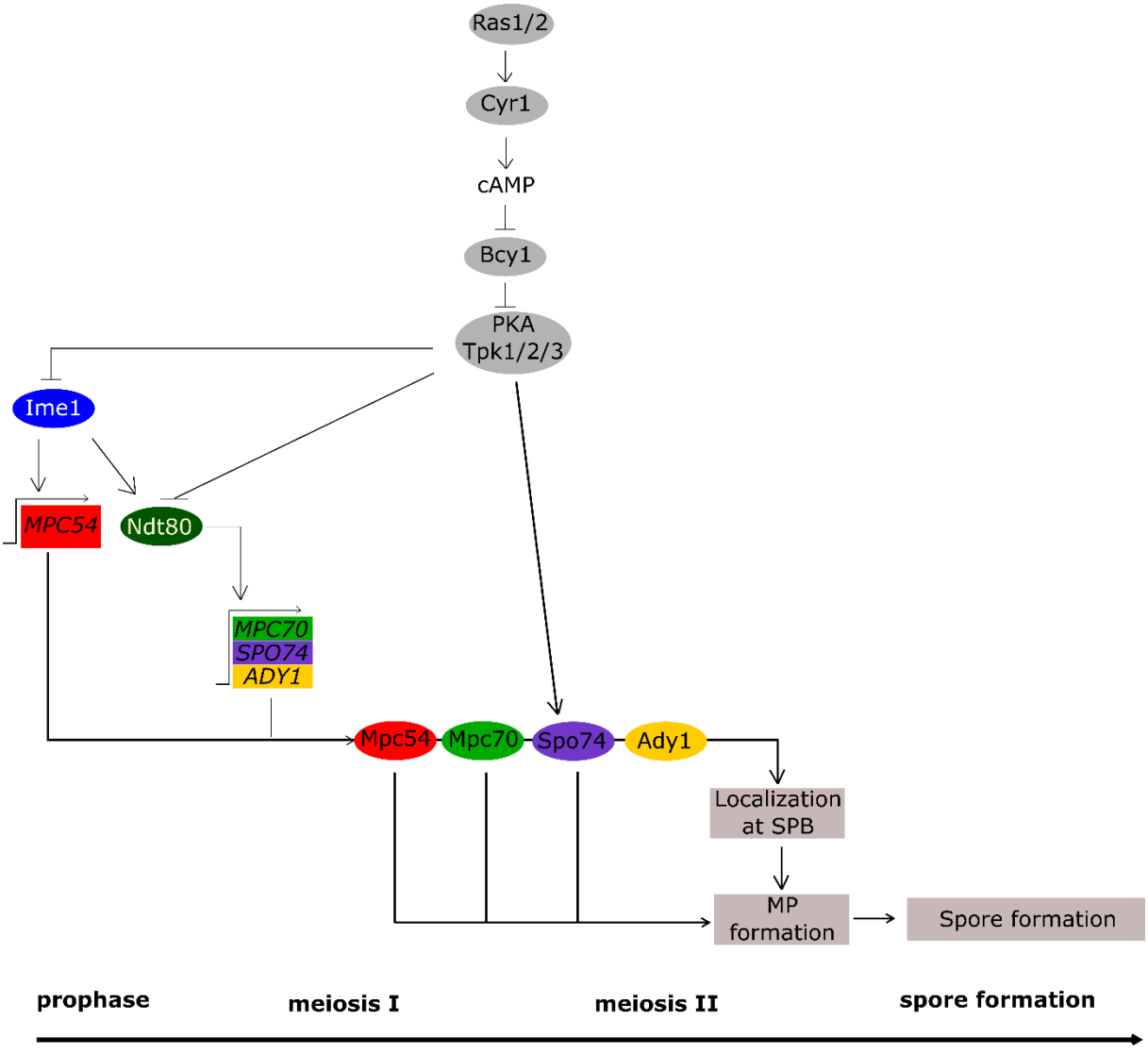

Figure 41: Model for the regulation of downstream targets by PKA during sporulation

PKA: *TPK1/2/3*. PKA affects the spore formation after commitment to meiosis. Mpc54, Mpc70, Ady1 are indirect targets of PKA, Spo74 is a direct target of PKA. More details about the figure are in the text.

Ime1 is the master activator of the early genes and leads to the expression of the early gene *MPC54* as well as Ndt80 transcription factor which is the activator of the middle genes like *MPC70*, *SPO74* and *ADY1*. The experiments demonstrated that mutants in the Ras/cAMP/PKA pathway caused changes in the abundance of all the essential MP

components which were reflected on the spore formation. Furthermore, I could show that PKA may affect directly Spo74 after commitment to meiosis, the experiments performed with modified consensus sites in Spo74 displayed changes in the sporulation behaviour. Finally, I could show that Ady1 which has a role in the activity of the MP (Deng and Saunders 2001; Jungbluth et al., 2012) and is phosphorylated by PKA *in vitro* (Ptacek et al., 2005), here it might be indirectly controlled by PKA *in vivo*. Both the regulation of MP protein levels as well as the protein level of Ady1 have an influence on the number of spores. This work demonstrated that PKA has several targets involved in spore formation and all of them influence the MP formation. Thus, PKA influences the MP formation on the level of the MP components synthesis and their ability to form a MP at the SPBs. This shows the complexity of PKA regulation on spore number control.

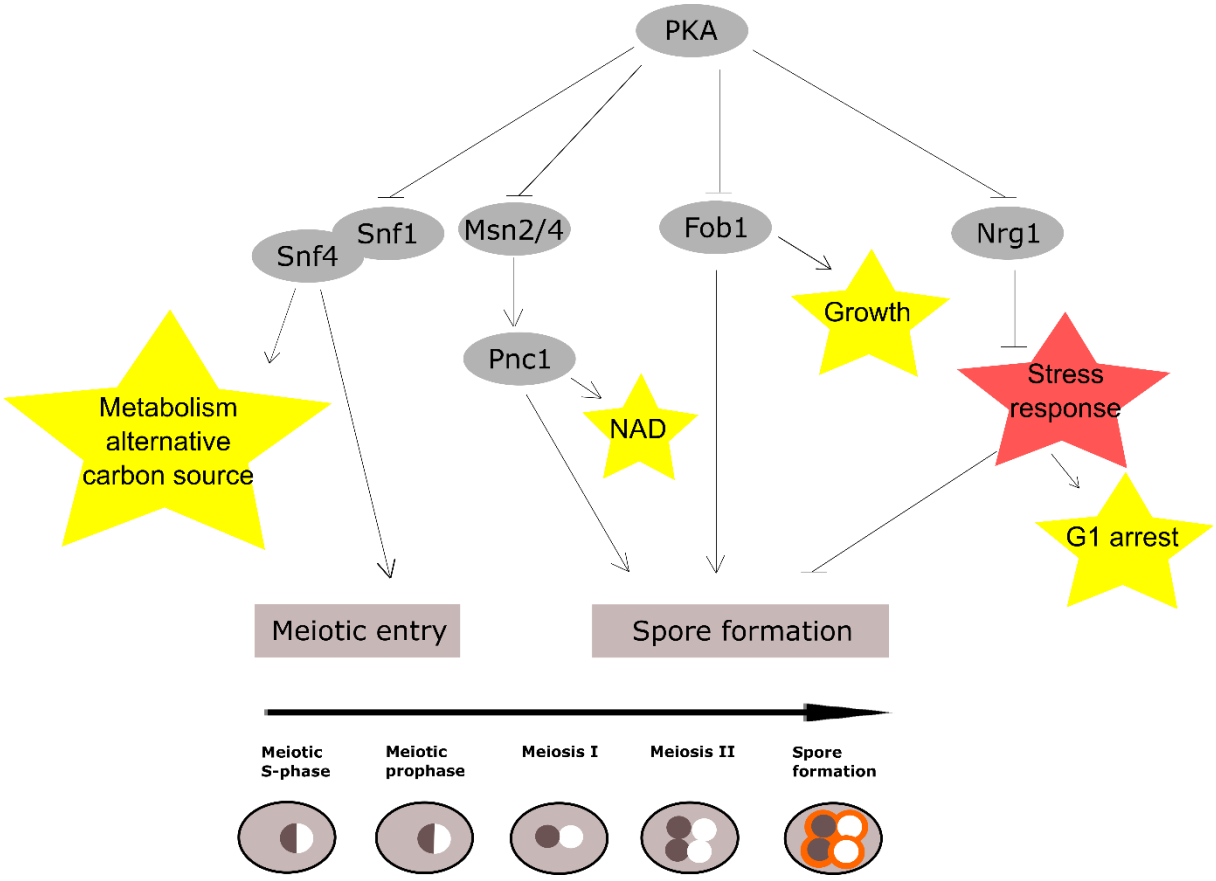

Figure 42: Model for the connection of PKA to other processes before and during sporulation

Snf1/Snf4 Sucrose non fermenting 1 and 4, Pnc1 Nicotinamidase, Fob1 Fork blocking less 1, Nrg1 Negative Regulator of Glucose-repressed genes 1. More details about the figure are in the text.

Part of the project wanted to answer the question if PKA regulates other processes. With my data I can assume that PKA can control negatively the transcription factor Nrg1 and the

nucleolar protein Fob1. Furthermore, PKA is involved in the production of NAD by regulating the nicotinamidase Pnc1. These targets of PKA are connected to spore formation. The metabolism of the alternative carbon sources is controlled by PKA as well by the complex Snf1-Snf4. The data shows that in this case cells are affected before to entry into meiosis (Fig. 41).

3.6 Outlook

The data suggested that PKA regulates downstream targets by affecting the abundance of MP components and Ady1. Intriguing is to know how PKA affects indirectly Mpc54, Mpc70 and Ady1. An interesting experiment would be to check if the regulation of the proteins occurs on transcriptional or protein level. Would be also newsworthy to know if Pnc1, Nrg1 and Fob1 effect the abundance or the activity of the MP components. NAD+ is a potential compound involved in spore number control. Its main role is to transfer electrons from a reaction to another one and in metabolism it has the function to transfer the energy of oxidized compounds to other processes. Would be interesting to see what is the effect of the overproduction of Pnc1 which potentially should increase the production of NAD+ on the spore numbers. PKA is embroiled in spore biogenesis because it regulates Smk1 MAP kinase responsible of the synthesis of mature spore wall (Wagner et al, 1999). Interesting is to know if PKA has other targets in the mid-late and late phase of spore formation. Another question to address could be how other pathways involved in sporulation like the target of rapamycin complex 1 (TORC1) signaling network would affect the spore numbers and the MP.

4. Materials

4.1 Chemicals, enzymes and kits

The chemicals used in this work were obtained from CARL ROTH GMBH & CO KG (Karlsruhe, DE), SIGMA-ALDRICH CHEMIE GMBH (Steinheim, D) and VWR INTERNATIONAL GMBH (Darmstadt, Germany).

Chemicals for the preparation of solutions, buffers and media have been prepared by the companies MERCK KGaA (Darmstadt, Germany), CARL ROTH GmbH & Co. KG (Karlsruhe, Germany), FLUKA (SIGMA-ALDRICH CHEMIE GmbH, Steinheim, D), SIGMA-ALDRICH CHEMIE GmbH (Steinheim, Germany) and VWR INTERNATIONAL GmbH (Darmstadt, Germany).

Restriction enzymes were ordered from FERMENTAS GmbH (St. LeonRot, Germany) and NEW ENGLAND BIOLABS Inc. (Frankfurt, Germany). “Generic 1 kb DNA Ladder” and “Generic DNA ladder mix” (FERMENTAS GmbH, St. Leon-Rot, Germany) were used as DNA size standards. RNAse A was obtained from METABION INTERNATIONAL AG (Martinsried, Germany).

“Phusion-DNA-Polymerase” was from FINNZYMES (Espoo, FIN).

Synthetic oligonucleotides were synthesized by METABION INTERNATIONAL AG (Martinsried, Germany).

For the preparation of plasmid DNA from *E. coli*, the "Plasmid Mini Kit" was used, for the extraction of DNA from agarose gels, the "QIAquick® Gel Extraction Kit" (QIAGEN GmbH, Hilden, D) or the E.Z.N.A. Gel extraction kit (OMEGA BIO-TEK, Norcross, USA) were used. Sequencing was carried out at SEQUENCE LABORATORIES GmbH (Göttingen, Germany).

Antibodies were produced by SANTA CRUZ BIOTECHNOLOGY Inc. (CA, USA), SIGMA-ALDRICH CHEMIE GmbH (Steinheim, D) and CALBIOCHEM (MERCK KGaA, Darmstadt, D).

Proteins and gel documentation systems by BIO-RAD LABORATORIES GmbH (Munich, Germany) were used.

All centrifugations of volumes up to 2 ml were carried out in centrifuges from HERAEUS HOLDING GmbH (Hanau, Germany): centrifugation at room temperature was carried out in a table centrifuge "Biofuge pico", all centrifugations at 4 ° C. in a table-type cooling centrifuge "Biofuge fresco". Centrifugation of volumes above 2 ml was carried out in a stand cooling centrifuge "4K-15" of SIGMA LABORZENTRIFUGEN GmbH (Osterode am Harz, Germany).

An inverse fluorescent microscope of the Axiovert 200M type (CARL-ZEISS AG, Oberkochen, Germany) was used for fluorescence microscopy. The microscope was equipped with a plan apochromat oil objective (NA 1.4; ZEISS), a digital camera (HAMAMATSU) and a GFP and DAPI filter set (ZEISS). Laboratory consumables were purchased from SARSTEDT AG & Co. (Nümbrecht, D).

4.1.1 Buffers and solutions

Table 1 shows used solutions and buffers. If not specified they were diluted with deionized water (dH_2O)

Table 2: Buffers and solutions used

Name	Composition
Alkaline lysis buffer	7,5% β-Mercaptoethanol 1,85 M NaOH
Blotting buffer	25 mM Tris 192 mM Glycine 20% methanol
Concanavalin-A	3 % (w/v) Concanavalin A
ECL- Solution	100 mM Tris HCl pH 9,2 2,5 mM 3- Aminophthalhydrazide (Luminol) 5,4 mM H_2O_2
Hoechst 33342 solution	1µm/ml Hoechst 33342 in 50% Glycerol
HU buffer (high-urea buffer)	7 M Urea 5% SDS 200 mM NaHPO4 pH 6,8 0,1 mM EDTA 0,1 % bromophenol 15 mg/ml DTT before using
LiPEG	mM EDTA 100 mM Lithium-Acetate

	40 % PEG4000 10 mM Tris/HCl pH 8
LiSORB	1 mM EDTA 100 mM Lithium-Acetate 1 M Sorbitol 10 mM Tris/HCl pH 8 (steril)
Loading buffer	10 mM Tris-HCl (pH 7.6) 0.03 % Bromophenol 0.03 % Xylene cyanol FF 60 % Glycerol 60 mM EDTA
Lysis buffer	2 % Triton X -100 1 % SDS 100 mM NaCl 10 mM Tris/Base pH 8 1 mM EDTA
Ponceau S	1% Ponceau S 1% acetic acid
Phusion buffer (10x)	200 mM Tris/HCl pH 8,8 100 mM KCl 100 mM (NH4)2SO4 40 mM MgSO4 1 % Triton X-100 1 mg/ml BSA
Running buffer	25 mM Tris 0,2 M Glycine 0,1 % SDS
Solution 1 (Plasmid isolation)	10 mM EDTA pH 8 50 mM Glucose 25 mm Tris/HCl pH 8
Solution 2 (Plasmid isolation)	0,2 M NaOH 1 % SDS
Solution 3 (Plasmid isolation)	5 M KOAc 2 M Acetic acid
TAE-Puffer	40 mM Tris-Acetate 20 mM Na-Acetate 2 mM EDTA pH 8,3
TBST	20 mM Na_2HPO_4 pH 7,1 100 mM NaCl 0,1 % Tween 20

4.1.2 Media

The used nutrient media were diluted with dH2O. For solid media, 2% agar was added. In the preparation of SC-solid medium, 4% agar and 2-fold concentrated SC- media were autoclaved separately and combined after autoclaving.

Table 3: Media used

Name	**Composition**
LB-medium (Lysogeny Broth)	0,5% Yeast extract 1% Trypton 1% NaCl
LFM medium (Low fluorescence)	10% Salt stock solution 0,1% Trace elements stock solution 0,1% Vitamins stock solution
Salt stock solution	5% $(NH_4)_2SO_4$ 1% KH_2PO_4 0,5% $MgSO_4$ 0,1% NaCl 0,1% $CaCl_2$
SC-4 medium (Synthetic complete without uracil, leucine tryptophan and histidine)	0,15% Yeast Nitrogen Base without Amino acids and Ammonium sulfate 0,5% Ammonium sulfate 0,2% Amino acid mixture 2% Glucose
SPO-Medium (Sporulation medium)	1% KOAc
Trace elements stock solution	0,05% H_3BO_4 0,004% $CuSO_4$ 0,01% KI 0,02% $FeCl_3$ 0,04% $MnSO_4$ 0,02% Na_2MoO_4 0,04% $ZnSO_4$
Vitamins stock solution	0,0002% Biotin 0,04% Calcium Pantothenate 0,2% Inositol 0,04% Niacin 0,02% Para-amino Benzoate 0,04% Pyridoxine HCl 0,04% Thiamine HCl
YP-medium (Yeast extract, Peptone)	1% Yeast extract 2% Tryptone Depending on the desired carbon source was added after autoclaving 2% Glucose 1% KOAc 2% Raffinose 3% Glycerol

Amino acid mixture

20g each

Alanine, arginine, asparagine, aspartate, cysteine, glutamine, glutamate, glycine, inositol, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, valine.

5g adenine

2 g para-aminobenzoic acid

The amount of 2 g histidine, uracil, tryptophan or 4 g of leucine was added to 36.7 g of the amino acid mixture to produce the corresponding SC-medium.

4.1.3 Antibiotics

The used antibiotics were prepared with double deionized water (ddH_2O). For the production of solid media, antibiotics were added after cooling of the agar to 60 ° C.

Table 4: Antibiotics used

Name	Stock solution	Final concentration	Origin
Ampicillin	100 mg/ml	100 µg/ml	CAL ROTH GMBH & CO KG (Karlsruhe, D)
ClonNAT (Nourseothricin)	200 mg/ml	100 µg/ml	WERNER BIO AGENTS (Jena, D)
G418 (Geniticin)	200 mg/ml	200 µg/ml	CAL ROTH GMBH & CO KG (Karlsruhe, D)
Kanamycin	100 mg/ml	100 µg/ml	CAL ROTH GMBH & CO KG (Karlsruhe, D)

4.2 Strains and Plasmids

4.2.1 *E. coli* strains

Table 5: *E. coli* strains used

Strain	Genotype	Reference
DH5α	F- *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* Φ80dlacZΔM15 Δ(lacZYA-	(Hanahan, 1983)

	argF)*U169, hsdR17(rK- mK+), λ–*	
TOP10	*F-, mcrA, Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1, araD139 Δ (araleu)7697 galU galK rpsL (StrR) endA1 nupG*	Invitrogen GmbH (Karlsruhe, D)

4.2.2 *S. cerevisiae* strains

The following table shows the *S. cerevisiae* strains used in this study. A PCR-based method for gene fusions and gene deletions was used (Janke et al., 2004). If no indicated, all used yeast strains derived from the genetic background of SK1. The genomic modifications in the yeast strains were checked by PCR. The mating type was tested either by SPO medium or by cross-linking with the mating type tester strains YR312 and YR320.

Table 6: Used *S. cerevisiae* strains

Strain	Genotype	Reference
ESM356	*MATa* *ura3-53 leu2Δ1 his3Δ200 trp1Δ63*	C. Taxis collection
ESM357	*MATα* *ura3-53 leu2Δ1 his3Δ200*	C. Taxis collection
SGY37	*MATa* *ura3-52::URA3-lexA-op-LacZ trp1 his3 leu2*	(Geissler *et al.*, 1996)
YAA129	*MATα* *lys2 leu2 ho::LYS2 ura3* *his3::IME2(P)-pTEV+-DIT1(T)::kanMX4::his3* *trp1::IME2(P)-pTEV+-DIT1(T)::kanMX4::trp1*	C. Taxis collection
YAA130	*MATa* *lys2 leu2 ho::LYS2 ura3* *his3::IME2(P)-pTEV+-DIT1(T)::kanMX4::his3* *trp1::IME2(P)-pTEV+-DIT1(T)::kanMX4::trp1*	C. Taxis collection
YAA146	*MATa /MATα* *lys2/lys2 leu2/leu2 ho::LYS2/ho::LYS2 ura3/ura3* *his3::IME2(P)-pTEV+-DIT1(T)::kanMX4::his3/* *his3::IME2(P)-pTEV+-DIT1(T)::kanMX4::his3* *trp1::IME2(P)-pTEV+-DIT1(T)::kanMX4::trp1/* *trp1::IME2(P)-pTEV+-DIT1(T)::kanMX4::trp1*	C. Taxis collection
YAM281	*MATa/MATα* YKS32 *MPC70-yeGFP::kanMX/MPC70-yeGFP::kanMX*	(Taxis *et al.*, 2005)
YCR77	*MATa/MATα* *ho::LYS2 his3 leu2 trp1 ura3/ho::LYS2 his3 leu2 trp1 ura3*	C. Taxis collection
YCR329	*MATa/MATα* *ura3-52 lys2 ho::LYS2/leu2delta ura3-52 lys2* *his3::IME2(P)-pTEV+-DIT1(T)::kanMX4::his3/* *his3::IME2(P)-pTEV+-DIT1(T)::kanMX4::his3* *trp1::IME2(P)-pTEV+-DIT1(T)::kanMX4::trp1/* *trp1::IME2(P)-pTEV+-DIT1(T)::kanMX4::trp1*	C. Taxis collection

YCR484	*MATa/MATα* *ura3*Δ */ura3*Δ his3Δ/his3Δ *leu2*Δ */leu2*Δ *trp1* Δ/*trp1*Δ *ho::hisG/ho::hisG* *MPC54/MPC54-6HA::spHIS5*	C. Taxis collection
YCR485	*MATa/MATα* *ura3*Δ */ura3*Δ his3Δ/his3Δ *leu2*Δ */leu2*Δ *trp1* Δ/*trp1*Δ *ho::hisG/ho::hisG* *MPC70/MPC70-6HA::spHIS5*	C. Taxis collection
YCR561	*MATa/MATα* *ura3*Δ */ura3*Δ *his3*Δ/*his3*Δ *leu2*Δ */leu2*Δ *lys2*Δ */lys2*Δ *trp1* Δ/*trp1*Δ *ho::LYS2/ho::LYS2* *SPO74/SPO74-9myc::hphNT1*	C. Taxis collection
YCT730	*MATa/MATα* YKS32 *MPC54-yeGFP::kanMX/MPC54-yeGFP::kanMX*	(Taxis *et al.*, 2005)
YDS152	*MATa/MATα* *ura3Δ /ura3Δ his3Δ/his3Δ leu2Δ /leu2Δ lys2Δ /lys2Δ trp1 Δ/trp1Δ ho::LYS2/ho::LYS2* *ADY1/ADY1-9myc::hphNT1*	C. Taxis collection
YKS32	*MATa/MATα* *lys2/lys2 ura3/ura3 leu2/LEU2 ho::hisG/ho::LYS*	(Knop and Strasser, 2000)
YMK725	*MATa/MATα* *lys2/lys2 ura3/ura3 leu2/LEU2 ho::his6/ho::LYS2 spo74*Δ *hphNT1:: spo74*Δ *hphNT1*	C. Taxis collection
YMJ36	*YKS32 MATa*	(Jungbluth *et al.*, 2012)
YMJ37	*YKS32 MATα*	(Jungbluth *et al.*, 2012)
YMJ68	*MATa/MATα* *ady1*Δ*::natNT2/ady1*Δ*::natNT2*	(Jungbluth *et al.*, 2012)
YMJ69	*MATa/MATα* *ady2*Δ*::hphNT1/ady2*Δ*::hphNT1*	(Jungbluth *et al.*, 2012)
YMJ143	*MATα* *cyr1*Δ*::hphNT1* pMJ15 [*CYR1 URA3 ARS/CEN*]	C. Taxis collection
YMJ144	*MATa* *cyr1*Δ*::hphNT1* pMJ15 [*CYR1 URA3 ARS/CEN*]	C. Taxis collection
YMJ145	*MATα* *cyr1*Δ*::hphNT1* pDS66 [$cyr1^{K1712A}$ *URA3 ARS/CEN*]	C. Taxis collection
YMJ146	*MATa* *cyr1*Δ*::hphNT1* pDS66 [$cyr1^{K1712A}$ *URA3 ARS/CEN*]	C. Taxis collection
YMM1	*MATα* *MPC70-yemGFP::KanMX cyr1*Δ*::hphNT1* pMJ15 [*CYR1 URA3 ARS/CEN*] Created by transformation of PCR product of primers MPC70-S2 and MPC70-S3 with pYM12 Monomeric as template into YMJ143. Checked by test PCR with hiskantag2 and MPC70-tag.	This work
YMM2	*MATa*	This work

	MPC70-yemGFP::KanMX cyr1Δ::hphNT1 pMJ15 [*CYR1 URA3 ARS/CEN*] Created by transformation of PCR product of primers MPC70-S2 and MPC70-S3 with pYM12 Monomeric as template into YMJ144. Checked by test PCR with hiskantag2 and MPC70-tag.	
YMM3	***MATα*** *SPO74-yemGFP::KanMX cyr1Δ::hphNT1* pMJ15 [*CYR1 URA3 ARS/CEN*] Created by transformation of PCR product of primers SPO74-S2 and SPO74-S3 with pYM12 Monomeric as template into YMJ143. Checked by test PCR with hiskantag2 and SPO74-tag.	This work
YMM4	***MATa*** *SPO74-yemGFP::KanMX cyr1Δ::hphNT1* pMJ15 [*CYR1 URA3 ARS/CEN*] Created by transformation of PCR product of primers SPO74-S2 and SPO74-S3 with pYM12 Monomeric as template into YMJ144. Checked by test PCR with hiskantag2 and SPO74-tag.	This work
YMM5	***MATa/MATα*** *MPC70-yemGFP::KanMX cyr1Δ::hphNT1/ MPC70-yemGFP::KanMX cyr1Δ::hphNT1* pMJ15 [*CYR1 URA3 ARS/CEN*] Created by crossing YMM1 and YMM2.	This work
YMM6	***MATa/MATα*** *SPO74-yemGFP::KanMX cyr1Δ::hphNT1/ SPO74-yemGFP::KanMX cyr1Δ::hphNT1* pMJ15 [*CYR1 URA3 ARS/CEN*] Created by crossing YMM3 and YMM4.	This work
YMM7	***MATα*** *MPC54-yemGFP::KanMX cyr1Δ::hphNT1* pMJ15 [*CYR1 URA3 ARS/CEN*] Created by transformation of PCR product of primers MPC54-S2 and MPC54-S3 with pYM12 Monomeric as template into YMJ143. Checked by test PCR with hiskantag2 and MPC54-tag.	This work
YMM8	***MATa*** *MPC54-yemGFP::KanMX cyr1Δ::hphNT1* pMJ15 [*CYR1 URA3 ARS/CEN*] Created by transformation of PCR product of primers MPC54-S2 and MPC54-S3 with pYM12 Monomeric as template into YMJ144. Checked by test PCR with hiskantag2 and MPC54-tag.	This work
YMM9	***MATα*** *MPC54-yemGFP::KanMX cyr1Δ::hphNT1* pDS66 [$cyr1^{K1712A}$ *URA3 ARS/CEN*] Created by transformation of PCR product of primers MPC54-S2 and MPC54-S3 with pYM12 Monomeric as template into YMJ145. Checked by test PCR with hiskantag2 and MPC54-tag.	This work
YMM10	***MATα***	This work

	ADY1-yemGFP::KanMX cyr1Δ::hphNT1 pMJ15 [*CYR1 URA3 ARS/CEN*] Created by transformation of PCR product of primers ADY1-S2 and ADY1-S3 with pYM12 Monomeric as template into YMJ143. Checked by test PCR with hiskantag2 and ADY1-tag.	
YMM11	***MATa*** *ADY1-yemGFP::KanMX cyr1Δ::hphNT1* pMJ15 [*CYR1 URA3 ARS/CEN*] Created by transformation of PCR product of primers ADY1-S2 and ADY1-S3 with pYM12 Monomeric as template into YMJ144. Checked by test PCR with hiskantag2 and ADY1-tag.	This work
YMM12	***MATa*** *ADY1-yemGFP::KanMX cyr1Δ::hphNT1* pDS66 [$cyr1^{K1712A}$ *URA3 ARS/CEN*] Created by transformation of PCR product of primers ADY1-S2 and ADY1-S3 with pYM12 Monomeric as template into YMJ146. Checked by test PCR with hiskantag2 and ADY1-tag.	This work
YMM13	***MATα*** *MPC70-yemGFP::KanMX cyr1Δ::hphNT1* pDS66 [$cyr1^{K1712A}$ *URA3 ARS/CEN*] Created by transformation of PCR product of primers MPC70-S2 and MPC70-S3 with pYM12 Monomeric as template into YMJ146. Checked by test PCR with hiskantag2 and MPC70-tag.	This work
YMM14	***MATa/MATα*** *MPC54-yemGFP::KanMX cyr1Δ::hphNT1/ MPC54-yemGFP::KanMX cyr1Δ::hphNT1* pMJ15 [*CYR1 URA3 ARS/CEN*] Created by crossing YMM7 and YMM8.	This work
YMM15	***MATa/MATα*** *ADY1-yemGFP::KanMX cyr1Δ::hphNT1/ ADY1-yemGFP::KanMX cyr1Δ::hphNT1* pMJ15 [*CYR1 URA3 ARS/CEN*] Created by crossing YMM10 and YMM11	This work
YMM16	***MATα*** *MPC70-yemGFP::KanMX cyr1Δ::hphNT1* pDS66 [$cyr1^{K1712A}$ *URA3 ARS/CEN*] Created by transformation of PCR product of primers MPC70-S2 and MPC70-S3 with pYM12 Monomeric as template into YMJ145. Checked by test PCR with hiskantag2 and MPC70-tag.	This work
YMM17	***MATα*** *SPO74-yemGFP::KanMX cyr1Δ::hphNT1* pDS66 [$cyr1^{K1712A}$ *URA3 ARS/CEN*] Created by transformation of PCR product of primers SPO74-S2 and SPO74-S3 with pYM12 Monomeric as template into YMJ146. Checked by test PCR with hiskantag2 and SPO74-tag.	This work
YMM18	***MATa/MATα***	This work

	MPC70-yemGFP::KanMX cyr1Δ::hphNT1/ MPC70-yemGFP::KanMX cyr1Δ::hphNT1 pDS66 [*cyr1^{K1712A} URA3 ARS/CEN*] Created by crossing YMM16 and YMM13.	
YMM19	***MATα*** *SPO74-yemGFP::KanMX cyr1Δ::hphNT1* pDS66 [*cyr1^{K1712A} URA3 ARS/CEN*] Created by transformation of PCR product of primers SPO74-S2 and SPO74-S3 with pYM12 Monomeric as template into YMJ145. Checked by test PCR with hiskantag2 and SPO74-tag.	This work
YMM20	***MATa/MATα*** *SPO74-yemGFP::KanMX cyr1Δ::hphNT1/ SPO74-yemGFP::KanMX cyr1Δ::hphNT1* pDS66 [*cyr1^{K1712A} URA3 ARS/CEN*] Created by crossing YMM19 and YMM17	This work
YMM21	***MATα*** *MPC54-yemGFP::KanMX cyr1Δ::hphNT1* pDS66 [*cyr1^{K1712A} URA3 ARS/CEN*] Created by transformation of PCR product of primers MPC54-S2 and MPC54-S3 with pYM12 Monomeric as template into YMJ146. Checked by test PCR with hiskantag2 and MPC54-tag.	This work
YMM22	***MATa*** *ADY1-yemGFP::KanMX cyr1Δ::hphNT1* pDS66 [*cyr1^{K1712A} URA3 ARS/CEN*] Created by transformation of PCR product of primers ADY1-S2 and ADY1-S3 with pYM12 Monomeric as template into YMJ145. Checked by test PCR with hiskantag2 and ADY1-tag.	This work
YMM23	***MATa/MATα*** *MPC54-yemGFP::KanMX cyr1Δ::hphNT1/ MPC54-yemGFP::KanMX cyr1Δ::hphNT1* pDS66 [*cyr1^{K1712A} URA3 ARS/CEN*] Created by crossing YMM9 and YMM21.	This work
YMM24	***MATa/MATα*** *ADY1-yemGFP::KanMX cyr1Δ::hphNT1/ ADY1-yemGFP::KanMX cyr1Δ::hphNT1* pDS66 [*cyr1^{K1712A} URA3 ARS/CEN*] Created by crossing YMM22 and YMM12	This work
YMM25	***MATa*** *nrg1Δ::hphNT1* Created by transformation of PCR product of primers NRG1-S1 and NRG1-S2 with pFA61-hphNT1 as template into YMJ36. Checked by test PCR with hiskantag2 and NRG1-KO	This work
YMM26	***MATα*** *nrg1Δ::hphNT1*	This work

	Created by transformation of PCR product of primers NRG1-S1 and NRG1-S2 with pFA61-hphNT1 as template into YMJ37. Checked by test PCR with hiskantag2 and NRG1-KO	
YMM27	*MATα* *yap1Δ::hphNT1* Created by transformation of PCR product of primers YAP1-S1 and YAP1-S2 with pFA61-hphNT1 as template into YMJ37. Checked by test PCR with hiskantag2 and YAP1-KO	This work
YMM28	*MATa/MATα* *nrg1Δ::hphNT1/nrg1Δ::hphNT1* Created by crossing YMM25 and YMM26	This work
YMM29	*MATa* *yap1::hphNT1* Created by transformation of PCR product of primers YAP1-S1 and YAP1-S2 with pFA61-hphNT1 as template into YMJ36. Checked by test PCR with hiskantag2 and YAP1-KO	This work
YMM30	*MATa/MATα* *yap1Δ::hphNT1/yap1Δ::hphNT1* Created by crossing YMM27 and YMM29	This work
YMM34	*MATa/MATα* *ura3-52 lys2 ho::LYS2/leu2delta ura3-52 lys2* *his3::IME2(P)-pTEV+DIT1(T)::kanMX4::his3/* *his3::IME2(P)-pTEV+-DIT1(T)::kanMX4::his3* *trp1::IME2(P)-pTEV+-DIT1(T)::kanMX4::trp1/* *trp1::IME2(P)-pTEV+-DIT1(T)::kanMX4::trp1* *natNT2::SCC1(P)::GFP-TEVdegF-SF3B-3HA/* *natNT2::SCC1(P)::GFP-TEVdegF-SF3B-3HA* Created by transformation of PCR product of primers BCY1-S1 and BCY1-S4 with pCR65 as template into YCR329	This work
YMM36	*MATa* *lys2 leu2 ho::LYS2 ura3* *his3::IME2(P)-pTEV+-DIT1(T)::kanMX4::his3* *trp1::IME2(P)-pTEV+-DIT1(T)::kanMX4::trp1* *NatNT2::Adh1(P)-GFP-TDegF-SF3B-3HA-BCY1* Created by transformation of PCR product of primers BCY1-S1 and BCY1-S4 with pCT289 as template into YAA130. Checked by test PCR with hiskantag2 and BCY1-KO.	This work
YMM37	*MATα* *lys2 leu2 ho::LYS2 ura3* *his3::IME2(P)-pTEV+-DIT1(T)::kanMX4::his3* *trp1::IME2(P)-pTEV+-DIT1(T)::kanMX4::trp1* *NatNT2::Adh1(P)-GFP-TDegF-SF3B-3HA-BCY1* Created by transformation of PCR product of primers BCY1-S1 and	This work

	BCY1-S4 with pCT289 as template into YAA129. Checked by test PCR with hiskantag2 and BCY1-KO.	
YMM40	*MAT**a*** *snf4Δ::hphNT1* Created by transformation of PCR product of primers SNF4-S1 and SNF4-S2 with pFA61-hphNT1 as template into YMJ36. Checked by test PCR with hiskantag2 and SNF4-KO	This work
YMM41	*MAT**α*** *snf4::hphNT1* Created by transformation of PCR product of primers SNF4-S1 and SNF4-S2 with pFA61-hphNT1 as template into YMJ37. Checked by test PCR with hiskantag2 and SNF4-KO	This work
YMM42	*MAT**a*** *fob1::hphNT1* Created by transformation of PCR product of primers FOB1-S1 and NRG1-S2 with pFA61-hphNT1 as template into YMJ36. Checked by test PCR with hiskantag2 and FOB1-KO	This work
YMM43	*MAT**α*** *fob1::hphNT1* Created by transformation of PCR product of primers NRG1-S1 and FOB1-S2 with pFA61-hphNT1 as template into YMJ37. Checked by test PCR with hiskantag2 and FOB1-KO	This work
YMM45	*MAT**a**/MAT**α*** *fob1Δ::hphNT1/fob1Δ::hphNT1* Created by crossing YMM42 and YMM43	This work
YMM46	*MAT**a**/MAT**α*** *lys2/lys2 leu2/leu2 ho::LYS2/ho::LYS2 ura3/ura3* *his3::IME2(P)-pTEV+-DIT1(T)::kanMX4::his3/* *his3::IME2(P)-pTEV+-DIT1(T)::kanMX4::his3* *trp1::IME2(P)-pTEV+-DIT1(T)::kanMX4::trp1/* *trp1::IME2(P)-pTEV+-DIT1(T)::kanMX4::trp1* *NatNT2::Adh1(P)-GFP-TDegF-SF3B-3HA-BCY1/* *NatNT2::Adh1(P)-GFP-TDegF-SF3B-3HA-BCY1* Created by crossing YMM36 and YMM37	This work
YMM50	*MAT**a*** *pnc1Δ::hphNT1* Created by transformation of PCR product of primers PNC1-S1 and PNC1-S2 with pFA61-hphNT1 as template into YMJ36. Checked by test PCR with hiskantag2 and PNC1-KO	This work
YMM51	*MAT**α*** *pnc1Δ::hphNT1* Created by transformation of PCR product of primers PNC1-S1 and PNC1-S2 with pFA61-hphNT1 as template into YMJ37. Checked by test PCR with hiskantag2 and PNC1-KO	This work

YMM52	*MAT**a**/MAT**α*** *pnc1Δ::hphNT1/pnc1Δ::hphNT1* Created by crossing YMM50 and YMM51	This work
YMM54	*MAT**a*** *lys2 leu2 ho::LYS2 ura3* *his3::IME2(P)-pTEV+-DIT1(T)::kanMX4::his3* *trp1::IME2(P)-pTEV+-DIT1(T)::kanMX4::trp1* *natNT2::Pcyc1::GFP-TEVdeg-SF3B-3HA-BCY1* Created by transformation of PCR product of primers BCY1-S1 and BCY1-S4 with pMJ8 as template into YAA129. Checked by test PCR with hiskantag2 and BCY1-KO.	This work
YMM55	*MAT**a**/MAT**α*** *snf4Δ::hphNT1/snf4Δ::hphNT1* Created by crossing YMM40 and YMM41	This work
YMM60	*MAT**a*** *lys2 leu2 ho::LYS2 ura3* *his3::IME2(P)-pTEV+-DIT1(T)::kanMX4::his3* *trp1::IME2(P)-pTEV+-DIT1(T)::kanMX4::trp1* *natNT2::Pcyc1::GFP-TEVdeg-SF3B-3HA-BCY1* Created by transformation of PCR product of primers BCY1-S1 and BCY1-S4 with pMJ8 as template into YAA130. Checked by test PCR with hiskantag2 and BCY1-KO.	This work
YMM64	*MAT**a**/MAT**α*** *ura3-52 lys2 ho::LYS2/leu2delta ura3-52 lys2* *his3::IME2(P)-pTEV+-DIT1(T)::kanMX4::his3/* *his3::IME2(P)-pTEV+-DIT1(T)::kanMX4::his3* *trp1::IME2(P)-pTEV+-DIT1(T)::kanMX4::trp1/* *trp1::IME2(P)-pTEV+-DIT1(T)::kanMX4::trp1* *natNT2::Pcyc1::GFP-TEVdeg-SF3B-3HA-BCY1/* *natNT2::Pcyc1::GFP-TEVdeg-SF3B-3HA-BCY1* Created by crossing YMM54 and YMM60	This work
YUK63	*MAT**a**/MAT**α*** YKS32 *SPO74-yeGFP::kanMX/SPO74-yeGFP::kanMX*	(Taxis *et al.*, 2005)

4.2.3 Plasmids

Table 7: Used plasmids

Plasmid	**Resistence/Insert/Auxotrophic marker**	**Reference**
B1819	Amp *LEU2 ARS/CEN ori*	C. Taxis collection
pAB1	Amp *SPO74 URA3 ARS/CEN ori*	C. Taxis collection
pAB4	Amp $spo74^{S253A\ S350A}$ *URA3 ARS/CEN ori*	C. Taxis collection
pAB7	Amp $spo74^{S253E\ S350E}$ *URA3 ARS/CEN ori*	C. Taxis collection

pAB8	Amp *GAL1(P)-GAL4AD-HA-spo74*$^{S253E\ S350E}$ *LEU2 2µ ori*	C. Taxis collection
pAB9	Amp *spo74*$^{S253A\ S350A}$*-GFP URA3 kanMX ARS/CEN ori*	C. Taxis collection
pAB10	Amp *spo74*$^{S253E\ S350E}$*-GFP URA3 kanMX ARS/CEN ori*	C. Taxis collection
pAB11	Amp *GAL1(P)-GAL4AD-HA-spo74*$^{S253A\ 3S350A}$ *LEU2 2µ ori*	C. Taxis collection
pCG347	Amp P_{TPI}*-3xRBD-GFP URA3 ARS/CEN*	(Leadsham and Gourlay 2009)
pCT289	Amp *PADH1-GFP-TDegF-SF3B-3HA natNT2*	(Taxis *et al.*, 2009)
pCR65	Amp *SCC1(P)::GFP-TEVdegF-SF3B-3HA natNT2*	C. Taxis collection
pDS181	Amp P_{ADH1}*- RFP-bPAC URA3 ARS/CEN ori*	C. Taxis collection
pFA61-hphNT1	Amp *hphNT1*	(Wach *et al.*, 1994)
pGREG599	Amp *GAL1(P)::HIS3::caGFP-CYC1(T)URA3 kanMX ori*	C. Taxis collection
pNRG1	Kan *NRG1 LEU2 2µ ori*	C. Taxis collection
pYAP1	Kan *YAP1 LEU2 2µ ori*	C. Taxis collection
pRTG1	Kan *RTG1 LEU2 2µ ori*	C. Taxis collection
pSUM1	Kan *SUM1 LEU2 2µ ori*	C. Taxis collection
pXBP1	Kan *XBP1 LEU2 2µ ori*	C. Taxis collection
pTEC1	Kan *TEC1 LEU2 2µ ori*	C. Taxis collection
pADY1	Kan *TEC1 LEU2 2µ ori*	C. Taxis collection
pJT4	Amp P_{ADH1}*- RFP-bPAC LEU2 ARS/CEN ori*	C. Taxis collection
pKS36	Amp *GAL1(P)-GAL4AD-HA-MPC70 LEU2 2µ ori*	(Knop and Strasser, 2000)
pKS40	Amp *GAL1(P)-GAL4AD-HA-MPC54 LEU2 2µ ori*	(Knop and Strasser, 2000)
pMAX2	Amp *SPO74-GFP URA3 kanMX ARS/CEN ori* Created by ligation of PCR product in the pGREG599 vector cut with NehI and AatII. Primers pGREG599_rec_Sac1_SPO74_Fw and Rec3_SPO74_Rw. Template Chromosomal DNA ESM356.	This work
pMJ9	Amp P_{CYC1}*-GFP-TDegF-SF3B-3HA natNT2*	(Jungbluth *et al.*, 2012)
pMJ16	Amp P_{SPS1}*-RAS2*^{G19V}*, URA3 ARS/CEN*	(Jungbluth *et al.*, 2012)
pMM5	Amp *GAL1(P)-LexA-MYC HIS3 2µ ori*	(Geissler et al., 1996)
pMM6	Amp *GAL1(P)-GAL4AD-HA 2µ ori*	(Geissler et al., 1996)
pRS316	Amp *URA3 ARS/CEN ori*	C. Taxis collection
pUK48	Amp *GAL1(P)-LexA-MYC-SPO74C HIS3 2µ ori*	C. Taxis collection
pUK49	Amp *GAL1(P)-GAL4AD-HA-SPO74 LEU2 2µ ori*	C. Taxis collection

pYM12	Amp *yemGFP KanMX*	C. Taxis collection

4.2.4 Oligonucleotides

Table 8: Oligonucleotides used

Name	**Sequence 5`-3`-direction**	**Use**
Hiskantag2	GCTGCGCACGTCAAGACTG	Test-PCRs
ADY1-S2	ATTATAATTCTACACAAAATTACATTTAA ATATTAATGGCTTTCTTCAAAATTCAATC GATGAATTCGAGCTCG	Production of YMM10, YMM11, YMM12 and YMM22
ADY1-S3	AGCGTTGATAAGAAACCAATATCTGAAG GAGTATGAATTTTCTCAAAAAGATTTTCG TACGCTGCAGGTCGAC	Production of YMM10, YMM11, YMM12 and YMM22
ADY1-Tag	ATTTTTGTGGGGTTCGACGG	Test-PCR YMM10, YMM11, YMM12 and YMM22
BCY1-KO	ACTGTGCTCGGATTCCGAC	Test-PCR YMM36, YMM37, YMM54 and YMM60
BCY1-S1	AACAAGCAGATTATTTTCAAAAGACAAC AGTAAGAATAAACGATGCGTACGCTGCA GGTCGAC	Production of YMM34, 36, 37,54 and 60
BCY1-S4	GAACAGTTGCAATTCGGCTTGCGATTCCT TGGGCAAAGAAGATACCATCGATGAATT CTCTGTCG	Production of YMM34, 36, 37,54 and 60
FOB1-KO	CTGTAGCATTTAGTCAAACGGG	Test-PCR YMM42 and YMM43
FOB1-S1	ATTTAACGATTGTGTGAGTGTGAATTTGT GCTGAGGATAACAATGCGTACGCTGCAG GTCGAC	Production of YMM42 and YMM43
FOB1-S2	TCACCTATGGTGACTCCTCCTTTCATTCT ATCCTACATATTATTAATCGATGAATTCG AGCTCG	Production of YMM42 and YMM43
MPC54-S2	CGAGCTCGAATTCATCGATTTAGGTTATA AACCTTAATTGTTTGGTAAGTGAACGAA ATGCTCA	Production of YMM7, YMM8, YMM9 and YMM21
MPC54-S3	GTTACTGACTCACCAAGACTTAAAGAAA ATGAACACGTTACAAATCGTACGCTGCA GGTCGAC	Production of YMM7, YMM8, YMM9 and YMM21
MPC54-tag	AGGAAGGCCCGAAAGATG	Test-PCR YMM7, YMM8, YMM9 and YMM21
MPC70-S2	TATGTATATATAGAATATTAAGGATTATA AAAGAATGTGTAGCTGTTGAGGTTCAAT	Production of YMM1, YMM2,

	CGATGAATTCGAGCTCG	YMM13 and YMM16
MPC70-S3	ACTCCGTATAAACAAAGCCAAAGACAAG TTCCGCACTCCATCAAGCGTACGCTGCAG GTCGAC	Production of YMM1, YMM2, YMM13 and YMM16
MPC70-tag	CATATTCTCTACTCCTTGTATC	Test-PCR YMM1, YMM2, YMM13 and YMM16
SPO74-S2	TATGCAAGACATGTATATATGATTAGTAG TTTTTTTTATTTACTTTCATTGATCAATCG ATGAATTCGAGCTCG	Production of YMM3, YMM4, YMM17 and YMM19
SPO74-S3	ATGCCCAGAAGAAGTAATGAAGAAATGT ATAGCTGGTCACGTAATCGTACGCTGCA GGTCGAC	Production of YMM3, YMM4, YMM17 and YMM19
SPO74-Tag	CCATAACTGGTGGAACACC	Test-PCR YMM3, YMM4, YMM17 and YMM19
NRG1-KO	GTGTCATCTTTCTACAGTCTGG	Test-PCR YMM25 and YMM26
NRG1-S1	CTCTCGACCAGCATATTACTACCCTTCGC AAACTTTCAGGCAATGCGTACGCTGCAG GTCGAC	Production of YMM25 and YMM26
NRG1-S2	ATAGTAGTACTGCTAATGAGAAAAACAC GGGTATACCGTCAATTAATCGATGAATTC GAGCTCG	Production of YMM25 and YMM26
PGREG599_rec_Sac1_SPO74_Fw	TTAACCCTCACTAAAGGGAACAAAAGCT GGAGCTCATCTTTTTTAGGCTCAAATCAA AAAAATG	Production of pMAX2
PNC1-KO	GAAAGAAAAAAAAAAAGGGGCAGG	Test-PCR YMM50 and YMM51
PNC1-S1	TTATCTATATCTTTGTTAGAAAGAATAAA ATACAGTACAAAAATGCGTACGCTGCAG GTCGAC	Production of YMM50 and YMM51
PNC1-S2	AGCCACCCTAGTTCATCAGGTTGAAGAA GTATTATTCAGCTCTTAATCGATGAATTC GAGCTCG	Production of YMM50 and YMM51
Rec3-SPO74-Rw	ATTCTTCACCTTTAGACATTCTCGAGGTC GAATTACGTGACCAGCTATAC	Production of pMAX2
SNF4-KO	GTATAAACATAAATGTATTCAAGAAGC	Test-PCR YMM40 and YMM41
SNF4-S1	TGCTGTGTTAGCATTAGGAGGAAGCGAA AAGGAAAATACATAATGCGTACGCTGCA GGTCGAC	Production of YMM40 and YMM41
SNF4-S2	CATTTATTTATAGTATGTACACAAAAATC TCATCGGCTCGTTTCAATCGATGAATTCG AGCTCG	Production of YMM40 and YMM41
YAP1-KO	CTTTTCTTTTTCTGGGTGCGG	Test-PCR YMM27

		and YMM29
YAP1-S1	CCACCCAAAACGTTTAAAGAAGGAAAAG TTGTTTCTTAAACCATGCGTACGCTGCAG GTCGAC	Production of YMM27 and YMM29
YAP1-S2	AAAAGTTCTTTCGGTTACCCAGTTTTCCA TAAAGTTCCCGCTTTAATCGATGAATTCG AGCTCG	Production of YMM27 and YMM29

4.2.5 Antibodies

Table 9: Primary antibodies.

Antibodies	**Diluition**	**Origin**
Mouse anti-HA	1:1000	SIGMA
Mouse anti-GFP	1:3000	SANTA CRUZ BIOTECHNOLOGIES (Santa Cruz, USA)
Mouse anti-Myc (9B11)	1:5000	CELL SIGNALING
Rabbit anti-RFP	1:1000	SANTA CRUZ BIOTECHNOLOGIES (Santa Cruz, USA)
Rabbit anti-TUB1	1:20000	Kind gift of M. Knop to C. Taxis

Table 10: Secondary antibodies.

Antibodies	**Diluition**	**Origin**
Goat anti-mouse	1:3000	Jackson Immuno Research/Dianova
Goat anti-rabbit	1:3000	SANTA CRUZ BIOTECHNOLOGIES (Santa Cruz, USA)

4.2.6 Rothiphorese gel

Table 11: Rothiphorese gel used

	10% resolving gel	**stacking gel**
Tris/HCL	3,75 ml 1M Tris/HCL pH8.8	2,5 ml 0.5 M Tris/HCL pH6.8
H2O	2,5 ml	6,1 ml
Rotiphorese gel 30	3,4 ml	1,3
10% SDS	100 µl	100 µl
TEMED	5 µl	10 µl

10% APS	50 µl	50 µl

5 Methods

5.1 Growth conditions

5.1.1 Cultivation of *E. coli*

E. coli cells were incubated in LB medium on a rotary shaker overnight (O/N). For plasmid selection, ampicillin was added at a final concentration of 100 µg / ml. Solid media contained 2% (w / v) agar.
For maintaining the culture, 800 µl of cell suspension were added to 50% glycerin and frozen at -80 ° C.

5.1.2 Cultivation of *S. cerevisiae*

S. cerevisiae cells were cultured in YP medium. For pre-cultures YPD (YP with 2% (w / v) Glc) was used. For selection by auxotrophic marker SC medium was used without the corresponding amino acids (L-histidine, L-Leucine, L-tryptophan and uracil). LFM medium was used for fluorescence microscopy experiments. Solid agar was added to 2% (w / v). For selection against antibiotic resistance, the corresponding antibiotics were used as indicated in Table 2. Solid media were incubated for two to three days at 30 ° C. in an incubator, liquid media were incubated at 30 ° C. on a rotary shaker. Cells first have grown on YPD plates for long-term culture, then scratched from the plate and stored in 1 ml of 15% glycerol solution at -80 ° C

5.1.2.1 Sporulation of *S. cerevisiae*

All experiments were performed in diploid yeast strains with SK1 genetic background. If no indicated, all mutations introduced into the genome were in homozygous form. For sporulation in liquid media, cells were streaked on YPD or SC- plates and incubated for two days at 30 ° C. Cells were then incubated on YP or SC- plates with 3% glycerin and incubated again for two days at 30 ° C. A pre-culture was inoculated with a large amount of cells in 50 ml YP or SC- + 2% Raff. The incubation was carried out for 20 h at 30 ° C. on a rotary

shaker. The next day, the OD600 was determined and a volume of cells corresponding to 2 OD600 was collected for sporulation culture, washed once with water and resuspended in SPO Medium. The SPO medium contained 1%, 0.1% or 0.01% KOAc.
For sporulation on solid media, 2% agar plates containing 1%, 0.1% and 0.01% KOAc were used. First, cells were streaked on YPD or SC- plates and incubated for two days at 30 ° C. in an incubator. Then, a small amount of cells was scratched from the plate and resuspended in dH2O. 50 µl of this cell suspension were added dropwise to each SPO plate and incubated at 30 ° C. for two days.
For sporulation in presence of bicarbonate, a solution of bicarbonate 0.1 mM was added after that the cultures sporulated for 6 hours in SPO liquid medium 0.1% KOAc (5 hours for the Mpc54-GFP). Successively flasks were sealed with adhesive tape and incubated at 30 ° C.

5.1.3 Measurement of optical density in liquid cultures

To measure the optical density (OD) as a measure of cell density, the WPA Biowave CO8000 Cell Density Meter (BIOCHROM LTD., Cambridge, UK) was used. The difference in the optical density of nutrient medium and culture was measured at a wavelength of 600 nm (ΔOD600). From a ΔOD600 of 1, the culture was diluted with medium and measured again. In E. coli cultures, a ΔOD600 of 1 corresponds to a cell density of approx. 2 × 108 cells / ml, whereas in S. cerevisiae is approximately 1.5 × 10 7 cells / ml.

5.1.4 Localization of Ady1

The investigation of the Ady1-GFP localization was described as follows. Sporulation of the strain YMM24 and controls was induced as described in chapter 5.1.1.2. The next day, the OD600 was determined and a volume of cells corresponding to 2 OD600 was collected for sporulation culture, washed once with water and resuspended in SPO Medium containing 0.1% KOAc. Subsequently cells were stuck in glass bottom dishes (MATTEK CORPORATION, Ashwell USA). For this purpose, 200 µl of a 3% concanavalin-A solution (SIGMA-ALDRICH CHEMIE GmbH, Steinheim, Germany) were pipetted onto the glass bottom in the middle of the bowls. After 5 min, the solution was removed and the glass bottom was washed with water. 200 µl of the cell culture were then pipetted onto the glass bottom. Cells were maintained for 5 minutes until they had sunk onto the glass bottom. The

medium was removed, washed once with KOAc 0.1% to remove non-adherent cells. Then, two ml of SPO medium containing the 0.1% of KOAc were added to the dishes.
The evaluation was carried out on a fluorescence microscope. An Axiovert 200M microscope from ZEISS was used. The microscope was equipped with a plan apochromat oil objective (NA 1.4; ZEISS), a digital camera (HAMAMATSU) and a GFP filter set (ZEISS). The images were recorded with the software Volocity (version 5.03, PERKIN ELMER, Waltham, Massachusetts, USA). After 4 hours transmitted light images (DIC) and GFP-Z stacks were recorded. For each experiment, between 100 and 200 cells were taken. DIC images and GFP-Z stacks were taken again every hour for other 4 hours. During the time course images of 100-200 cells were taken every hour. The images were evaluated using the software ImageJ (version 1.44p). For this purpose, a maximal projection of the individual GFP stacks was applied, and Ady1-GFP was localized in the cell nucleus in some cells for each time point.

5.1.5 Localization of the MP components

The investigation of the localization of the MP components Mpc54-GFP, Mpc70 and Spo74-GFP was described as follows. Sporulation of the strains YMM23, YMM18, YMM20 and the respective controls was induced as described in chapter 5.1.1.2. The next day, the OD600 was determined and a volume of cells corresponding to two OD600 was collected for sporulation culture, washed once with water and resuspended in SPO Medium containing 0.01, 0.1 and 1% KOAc. The evaluation was carried out on a fluorescence microscope, description of the microscope is found in chapter 5.1.4. After 4 hours (3 hours for Mpc54-GFP) transmitted light images (DIC) and GFP-Z stacks were recorded. For each experiment, between 100 and 200 cells were taken. DIC images and GFP-Z stacks were taken again every hour for other 4 hours. During the time course images of 100-200 cells were taken every hour. The images were evaluated as described in chapter 5.1.4 and Mpc54-GFP, Mpc70-GFP and Spo74-GFP were localized to the SPBs for each time point.

5.1.6 Localization of Ras

The investigation of the RBD-GFP localization was described as follows. Cultivation of the yeast cells was induced as described in chapter 5.1.2. in TC flasks and in darkness. The next day, Once the vegetative state was reached, microscope pictures were taken. The evaluation was carried out on a fluorescence microscope. as described in chapter 5.1.4. Afterwards cells

were immediately subjected to blue light. After 3 hours transmitted light images (DIC) and GFP-Z stacks were recorded. For each experiment, between 100 and 200 cells were taken. The images were evaluated using the software ImageJ (version 1.44p). For this purpose, a maximal projection of the individual GFP stacks was applied, and RBD-GFP was localized in the cell nucleus and plasma membrane in some cells. To check the RBD-GFP signal in sporulation cells a volume corresponding to 2 OD600 of the same yeast cells which have reached the vegetative state in darkness was collected for sporulation culture, and treated as described in chapter 5.1.4. Stucked cell in glass bottom dishes started the sporulation in darkness. After 2 hours transmitted light images (DIC) and GFP-Z stacks were recorded. For each experiment, between 100 and 200 cells were taken. Afterwards, blue light was switched on and cells were subjected for one hour to an intensity of 30 micromol. DIC images and GFP-Z stacks were then taken again. The images were evaluated using the software ImageJ (version 1.44p) as described in chapter 5.1.4. RBD-GFP was localized in the cell nucleus and plasma membrane in some cells

5.2 Hoechst staining of *S. cerevisiae* cells

Sporulated cells were scratched from SPO plates and put in 70% ethanol and fixed in ethanol at room temperature (RT) for 1 h. Cells were then centrifuged at 400 g for 3 min. The ethanol supernatant was removed and 50% glycerol solution with 1 µg / ml Hoechst 33342 were added for staining the DNA. After incubation at RT for one hour, cells were either stored at -20 ° C. or subjected directly to a microscopic analysis

5.3 Evaluation of sporulation and determination of sporulation efficiency

For the evaluation of the sporulation and determination of the sporulation efficiency, cells stained with Hoechst 33342 were used. For each experiment, more than 200 cells were taken. Transmitted light images (DIC) and DAPI-Z stacks were recorded. Each experiment was repeated at least twice with at least two mutually independent transformers. A 63 x Axiovert 200M microscope from ZEISS was used. The microscope was equipped with a plan apochromat oil objective (NA 1,4; ZEISS), a digital camera (HAMAMATSU) and a DAPI filter set (ZEISS). The images were recorded with the software Volocity (version 5.0.3). A maximum projection of the DAPI-Z stacks, which was superimposed with the corresponding DIC image, was created with the software ImageJ (version 1.44p) to evaluate the images. The

various cell species used for evaluation are shown in Figure 10. The sporulation efficiency was calculated using the following formula: [(% tetrads * 4) + (% triads * 3) + (% dyads * 2) + % monads] / 4

5.7 Transformation of DNA

5.7.1 Transformation of chemocompetent *E. coli* cells

Chemocompetent *E. coli* cells were prepared as described in Hanahan, 1983. The transformation was carried out as described in Pope and Kent, 1996.

5.7.2 Transformation of electrocompetent *E. coli* cells

An aliquot of electrocompetent cells was thawed on ice and transferred to a cooled electroporation cuvette with a 1 mm gap width. 1 µl of plasmid DNA was added to the cells. Bubbles were removed by tapping the cuvette. Electroporation was performed with the following settings: voltage: 2.15 kV, resistance 200 Ω, capacity 25 µF. The time constant was between 4.6 and 4.8 ms. After electroporation, cells were transferred to 1 ml of cooled LB medium and incubated at 37 ° C. for 1 h. Cells were then pelleted at 2300 g for 3 min, plated onto LB-AMP plates and incubated at 37 ° C.

5.7.3 Transformation of *S. cerevisiae*

S. cerevisiae cells were transformed through the lithium acetate (LiOAc) method described in Knop et al., 1999. 50 ml of YPD were inoculated from an O/N culture to an OD600 of 0.2 and incubated to 30 ° C. to an OD600 of 0.8. Cells were pelleted at 400 g for 3 min and washed once with 50 ml of water. After a further pelletization step, cells were resuspended in 20 ml of LiSORB. Cells were again centrifuged and resuspended in 450 µl of LiSORB, in addition, 50 µl of previously boiled and cooled salmon sperm DNA were added to the cells. 50 µl aliquots were either stored at -80 ° C. or used directly for transformation. For transformation, about 5-10 µl plasmid DNA was added to 50 µl, ice-thawed, competent cells and mixed. This was followed by a period of 20 minutes incubation at RT. Cells were then incubated for 15 min at 42 ° C. in a heating block. After incubation, 1 ml of YPD was added to the cells and centrifuged for 3 min at 400 g. When selected for antibiotic resistance, the supernatant was

removed and 1 ml of YPD was added again. This was followed by a centrifugation step at 400 g for 3 min, the supernatant was removed, and cells were incubated in 3 ml of YPD for 4-6 h at 30 ° C before plating on antibiotic-containing YPD plates. When selected for auxotrophy complementation, cells were plated on appropriate SC plates immediately after the first wash step.

5.7.4 Experiments with photoactivated adenylyl cyclase and blue light

bPAC plasmid was transformed in yeast competents and plated on specific SC plates as described in chapter 5.7.3. Yeast cells with the bPAC were kept in darkness in a box. Sporulation in liquid medium was induced as described in chapter 5.1.2.2. with the only difference that cultures in TC flasks for cultures sporulated for three hours in darkness. Afterwards blue light with specific intensities was switched on for 41 hours. For the experiment described in fig. 11 blue light was switched on for just 4 hours. For the experiment to check the effect of the photoactivated adenylyl cyclase on the Ras activity in vegetative cells, cells reached the stationary phase in darkness. Afterwards blue light was switched on.

5.7.5 Yeast two hybrids by ONPG assay

The ONPG assay was essentially performed as described previously (6). One absorbance unit of yeast cells per sample was used, except for Spo74-Spo74 where only 0.05 A was used due to the strong β-gal activity found with these interaction pairs. Cells were pelleted in Eppendorf® tubes and resuspended in 500 µL Z-buffer. Ten microliters chloroform and 15 µL 0.2% sodium dodecyl sulfate (SDS) were added, and the tubes were vortex mixed briefly. After a 5-min incubation at 28°C, 100 µL ONPG solution were added. The reaction was stopped after 10 min for Spo74-Spo74 and after 25 min for all other samples by adding 250 µL 1 M Na2CO3. Absorbance was determined at 420 nm. Results were normalized against cell density and incubation time.

5.8 Isolation and manipulation of DNA

5.8.1 Plasmid Isolation from *E. coli*

Preparation of plasmid DNA from E. coli was carried out by alkaline lysis. 1.5 ml of a culture was pelletized for 2 min at 2300 rpm. After removal of the supernatant, cells were resuspended in 100 µl of solution 1. Then, 200 µl of solution 2 was added and mixed. The lysis was carried out for 5 min at RT. After addition of 150 µl of solution 3, lysed cells were incubated on ice for 5 min. 400 µl of the supernatant was transferred to a new reaction vessel, 1 ml of ice-cold 100% ethanol was added and the mixture was incubated at -20 ° C. for 10 min. The batches were then centrifuged at 13000 rpm for 10 min. After removal of the supernatant, the precipitated DNA was dissolved in 20 µl of ddH2O with 1 µl of RNAse.
Alternatively, the plasmid mini kit from QUIAGEN GmbH (Hilden, Germany) was used. The procedure was carried out according to recommendations of the manufacturer.

5.8.2 DNA Isolation from *S. cerevisiae*

A large amount of cells were scratched from YPD plate and placed in 500 µl of extraction buffer containing 200 µl of glass beads (0.25-0.5 mm in diameter). 500 µl of phenol / chloroform / isoamyl alcohol (25: 24: 1) were then added and the mixture was shaken for 10 minutes at 4 ° C. on a Vibrax Basic shaker (IKA). This was followed by a centrifugation step for 10 minutes at 13000 rpm. 400 µl of the aqueous phase was transferred to a new reaction tube and 1 ml of ice-cold 100% ethanol was added. The batches were incubated for 10 min at -20 ° C. After centrifugation at 13000 rpm for 10 min, the pellet was dried slightly and resuspended in 20 µl of ddH2O with 1 µl RNAse A.

5.8.3 DNA amplification by polymerase chain reaction

For the preparation of PCR products for gene fusions, the primers were designed as described in Janke et al., 2004. The PCR blocks Primus 25 advanced or Primus 96 advanced (PEQLAB BIOTECHNOLOGIE GMBH, Erlangen, Germany) were used:

For the amplification of the PCR products, the following PCR protocol was used:

10µl 10x PHUSION buffer (40mM MgSO4)
3,5µl 10mM dNTPs
6,5µl 10µM primer 1
6,5µl 10µM primer 2

69,5 H20
2µl DNA
2µl PHUSION polymerase 1:2000 (Hot start)

The following PCR program was used:

98 °C 30s
Open the lid
Add the polymerase
Close the lid

10 cycles
58 °C 45s
68 °C Elongation (1 min/kb)
98 °C 30s

10 cycles
56 °C 45s
68 °C Elongation (1 min/kb + 10 s/cycle)
98 °C 30s
8 °C Storage

To concentrate the DNA from the PCR batch, the PCR products were precipitated with ethanol. For this purpose, 1/10 volume of ammonium acetate (3 M) and 2.5 volumes of 100% ethanol were added to the PCR batch and incubated for 10 min at -20 ° C. After 10 minutes centrifugation step at 13,000 rpm, the cell pellet was dried at RT and resuspended in 20 µl of dH2O

For test PCR the following PCR protocol was used:

10µl 10x Taq buffer
2µl 10mM dNTPs
10µl 10µM primer 1
10µl 10µM primer 2

68µl H2O
1µl DNA
0,2µl Taq polymerase

The following PCR program was used:

35 cycles
98 °C 30s
58 °C 45s
72 °C 1 min/1000 bp
98 °C 30s
8 °C Storage

5.8.4 Gel electrophoresis separation of DNA fragments

DNA fragments were separated in 1% TAE agarose gels at a voltage of 130 V and a current intensity of 300 mA for 40-60 min. The "GeneRuler ™ DNA Ladder Mix" (FERMENTAS GmbH, St. Leon-Rot, Germany) served as a standard for size. Agarose gels were incubated in 0.5 µg / ml of ethidium bromide solution for 10 min and then decolorized for 10 min in a water bath. The DNA bands were detected and photographed under UV light at a wavelength of 254 nm using a GelDoc XR system and the software Quantity One 1-D (BIO-RAD LABORATORIES GmbH).

5.8.5 Extraction of DNA from agarose gels

DNA was prepared according to the protocol of E.Z.N.A. Gel extract kit (OMEGA BIO-TEK, Norcross, USA) or the QIAquick® Gel Extraction Kit (QIAGEN GmbH) from 1% TAE agarose gels.

5.8.6 Enzymatic restriction of DNA

Restriction enzymes used were obtained from the companies FERMENTAS (St. Leon-Rot, Germany) and NEW ENGLAND BIOLABS INC. (Ipswich, UK). The restriction was made under conditions recommended by the manufacturers.

5.8.9 In vivo ligation in *S. cerevisiae*

The in vivo ligation of DNA fragments in S. cerevisiae was carried out according to the method described in Jansen et al., 2005. The yeast strain ESM356 was used for in vivo ligations.

5.8.10 Chromosomal integration of DNA in *S. cerevisiae*

Chromosomal integration of DNA in S. cerevisiae was performed according to the method described in Janke et al., 2004. The selected PCR conditions can be found in section 5.8.3. Details on primers used are given in Table 5 and Table 7.

5.8.11 DNA sequencing

To prepare the sequencing, 4 µl of plasmid DNA (from the alkaline lysis) were mixed with 9 µl of Tris / HCl (25 mM, pH 8) and 2 µl of oligonucleotide solution (10 µM). Sequencing was carried out as "Extended Hotshot" by Sequence Laboratories Göttingen GmbH (Göttingen, Germany).

5.9 Protein analysis and detection

5.9.1 Preparation of protein extracts from *S. cerevisiae*

The preparation of protein extracts from *S. cerevisiae* was performed by TCA precipitation. At the respective times, a quantity of cells corresponding to an OD600 of 2 was taken from the cultures. The cell suspension was pipetted into ice-cooled reaction vessels containing 150 µl of alkaline lysis buffer. After 10 minutes incubation of the samples on ice, 150 µl of 55% (w / v) trichloroacetic acid (TCA) was added and incubated again on ice for 10 min. This was followed by a centrifugation step at 13000 g for 10 min. The supernatant was discarded that pellet was resuspended in 100 µl of urea buffer (HU). The protein extracts were used either directly for denaturing gel electrophoresis (SDS-PAGE), or stored at -20 ° C.

In order to determine the protein quantity of the MP proteins formed during the meiosis, protein extracts were resuspended in 100 µl of HU buffer. Subsequently, 10 µl of the protein extract were combined into a new reaction vessel from each time point and separated by SDS-PAGE. The combined samples reflect the total amount of meiotic plate proteins formed during meiosis. Quantification of the proteins was performed by normalization of the bands with the respective loading control Tub1 bands.

5.9.2 Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

Protein were separated by their relative molecular mass under denaturing and discontinuous conditions. Self-made SDS gels were used (Table tot). The electrophoresis was carried out in a mini-Protean 3 apparatus (BIORAD) filled with electrophoresis buffer. Electrophoresis was started with a voltage of 150V. When the running front reached the separation gel, a voltage of 200 V was set (current 30 mA, power 6 W). The size standard used was Pageruler ™ Prestained Protein Ladder (FERMENTAS, St. Leon-Red, D).

5.9.3 Blotting

For blotting proteins, they were transferred from the separation gel to a Protran® nitrocellulose membrane (WHATMAN GmbH). This was previously soaked in BlottingPuffer. The following setup was used: On the black side of the transfer cassette were placed from bottom to top: fiber pad, WHATMAN® filter paper (WHATMAN GMBH), separation gel, Protran® nitrocellulose membrane, WHATMAN® filter paper, fiber pad. The cassette was then inserted into the MiniTransBlot® Electrophoretic Transfer Cell. The blotting was carried out at a voltage of 20 V overnight.

5.9.4 Immunodetection of proteins

After blotting, proteins were stained with Ponceau S solution to check the blotting efficiency, which was subsequently removed by washing with dH2O. For the binding of the antibodies to the membrane, this was first incubated in TBST with 5% (w / v) skimmed milk powder for 1 hour at RT. The membrane was then washed twice with TBST. The primary antibody was given in appropriate dilution (see Table 8) in 5% TBST containing 5% skimmed milk powder. The membrane was incubated in a 50 ml reaction vessel for 1 hour at RT on a roller incubator

(SRT6 scooter (Bibby Scientific Limited)). The membrane was then washed three times with TBST for 3 min. The secondary antibody was given in appropriate dilution (see Table 9) in TBST with 5% (w / v) skimmed milk powder. The incubation was carried out in a 50 ml reaction vessel for 1 hour at RT on a roller incubator. After incubation, the membrane was washed 6 times for 3 min with TBST.

The proteins were detected by the ECL method (Tesfaigzi et al., 1994). The ECL solution was prepared by itself (see Table 1). The excess liquid was removed from the membranes by draining, then about 5 ml of ECL solution was added to the membrane and incubated for 1 min at RT. The chemiluminescence was detected with a Chemostar Professional apparatus (INTAS SCIENCE IMAGING INSTRUMENTS GMBH, Göttingen, Germany), the detection time was dependent on the signal strength approximately 3-10 min.

To remove the antibodies from the membrane, the membrane was incubated for 30 min at 60 ° C. in a solution of 2% SDS, 100 mM Tris / HCl (pH 6.8) and 0.1 M β-mercaptoethanol. After 3 three-minute wash steps, the membrane was blocked at 5% (w / v) for 1 h at room temperature in TBST. All further steps were as described above.

5.9.5 Fluorescence microscopy

A reverse fluorescence microscope of the Axiovert 200M type (CARL-ZEISS AG, Oberkochen, Germany) was used. This was equipped with a CCD camera 1394 ORCA-ERA (HAMAMATSU, Herrsching am Ammersee, Germany). Only a 63x Plan Apochromat oil objective (NA 1.4; ZEISS) was used. IMMERSOL ™ 518 F from ZEISS was used as an immersion oil. The configuration of the microscope and camera was performed by the program Volocity (PERKINELMER LIFE and ANALYTICAL SCIENCES, INC., Rodgau-Jügesheim, D). GFP fluorescence was used with an enhanced GFP filter cube (excitation 488 nm, emission 509 nm), fluorescence from Hoechst 33342 with a DAPI filter cube (excitation 350 nm, emission 461 nm). Image processing was performed with the software ImageJ, Version 1.44p (Collins, 2007)

5.10 Software

Microscope control: Volocity version 5.0.3 (PERKIN ELMER, Waltham, Massachusetts, USA)

Planning of DNA manipulations in silico: Clone Manager 9 Basic Edition (SCIENTIFIC & EDUCATIONAL SOFTWARE, Cary USA).

Verification of Sequencing: Sequencher® Software (Demo Version, GENE CODES CORPORATION, Ann Arbor, MI USA).

Evaluation of microscopy data: ImageJ (version 1.44p, open source, Collins, 2007)

Alignment of protein sequences: Clustal X (version 2.1, Jeanmougin et al., 1998)

6 Literature

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7 Curriculum vitae

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Education:

01.02.12- today

PhD in Molecular Genetics
Philipps University Marburg, GER
Focus: genetics, molecular biology, eukaryotic cell, cloning, gene deletion, protein depletion, fluorescence microscope.

Dissertation Title: „Meiotic targets of the Ras/cAMP/PKA pathway during the regulation of spore formation in *Saccharomyces cerevisiae"*.

2006-2010

Master research degree in Biological Science (100/110)
University of Calabria, IT
Focus: genetic variability, DNA extraction, PCR.

Master Thesis Title: „Genetic variability in the species *Dianthus guliae*".

1999-2006

Bachelor degree in Biological Science (88/110)
University of Calabria, IT
Focus: cyto-physiology of plants, environmental bio-indicators, epiphyte and morphometric analysis.

Bachelor Thesis Title
„Monitoring of the lower limits of seagrass *Posidonia oceanica* by using the balisage technique".

1994-1999

High school diploma
Istituto Tecnico Industriale Statale ITIS, Crotone (IT)

Work experiences:

01.2011-10.2011	**Food Analysis,** Agriparadigma, Ravenna (IT) Product analysis and nutrional analysis of food, seed and flour, oils and fats and grass for animal nutrition.
05.2009-06.2010	**Diagnostic analysis,** Italsistemi, Crotone (IT) Microbiological analysis, HCV, ELISA, PCR.
06.2007-05.2009	**Research**, Italsistemi, Crotone (IT) Purification of proteins, western blotting, SDS page, fluorimeter, HeLa cells.
01.2007-05.2007	**Clinical analysis**, Tomasso, Crotone (IT) Hematology, immunology E.I.A., clinical biochemistry, microbiological and physical chemical analysis of urine.

Conferences:

10.2014	DFG International Symposium "Intra and Intercellular Transport and Communication IITC", Marburg (GER)
29.08.13 – 03.09.13	International Conference on Yeast Genetics and Molecular Biology, Frankfurt am Main (GER)

Publications:

Svetlana Usherenko, Hilke Stibbe, Massimiliano Muscò, Lars-Oliver Essen, Ekaterina A Kostina and Christof Taxis. **Photo-sensitive degron variants for tuning protein stability by light.** BMC Systems Biology 2014, 8:128

Workshops:

15.02.13 and 16.03.13	Writing Scientific Publications in the Life and Natural Science, Marburg University Research Academy (MARA), Marburg (GER)
25.02.2013	Project Management in Biotech Industries, Marburg University Research Academy (MARA), Marburg (GER)
06.2015	Bioimaging suite to obtain high quality microscopy, University of Exeter, Exeter (UK)

Training courses:

06.2006 - 07.2007	Development of biologically active peptides for the diagnosis and therapy of the neoplastic proliferations of B cells, Crotone (ITA)

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Acknowledgments

First of all I would like to say thanks to Prof. Dr. Hans-Ulrich Moesch for giving me the possibility to make my doctorate in his laboratory and for accepting to be the second reviewer of my thesis.

Thanks to Prof. Dr. Uve Mayer and Prof. Dr. Ralf Jakob for the willingness to attend my examining committee and for the comments and discussions during the retreats and seminars within the GRK 1216.

A great thank you to my boss and first reviewer PD Dr. Christof Taxis that made me realize the dream to do a PhD abroad. Thank you for encouraging me during the initial period when I needed and also for stimulating me to work hard and study to enlarge my knowledge. All of this contribuited a lot to get my doctorate.

Thanks to all the members of Moesch and Boelker groups especially Bernardo and Vivek for the funny moments together, Christian for the constant technical assistance during the work and Esmeralda for being a great friend always present in all!

Thanks to Alex, Pramod, Vikram, Reda, Elisa and all the people that who more who less made part of my life here in Germany. Thanks also to the people who supported me from Italy, Aldo (actually from England), Salvatore, Marika, of course my family and all my friends.

Thanks also to me because I made possible the impossible with my patience and constancy.

Last but not least, I would like to thank the DFG Graduate Research Fellowship 1216 for the financial support and the opportunity to attend important and interesting seminars, retreats and scientific workshops.

GRAZIE